ADIPOSE TISSUE DERIVED IL-6: REGULATION OF RELEASE AND PHYSIOLOGICAL SIGNIFICANCE

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Abstract

Signals derived from adipose tissue, such as interleukin-6 (IL-6), might explain the link between obesity and type 2 diabetes and cardiovascular disease. Significant amounts of IL-6 are released by adipose tissues. However, whether adipocytes within the tissue are responsible for the release, how this release is regulated and what the paracrine/autocrine effects of this adipokine might be are not known.

Rodent obesity, as with human obesity, is associated with elevated plasma IL-6 concentrations. In both lean and obese animals, the epididymal fat depot released more IL-6 than did the subcutaneous, but was only significantly different in the obese [lean; 2.8 (2.5–5.9) pg/ml vs. obese; 8.6 (4.6–24.5) pg/ml, median (interquartile range), p=0.0001]. Furthermore, whereas subcutaneous adipose tissue from both lean and obese animals released comparable levels of IL-6, epididymal IL-6 production increased significantly in obesity.A large proportion (up to 50%) of adipose tissue IL-6 secretion, from both depots, was constitutive. However, following stimulation, the largest change in the rate of production of IL-6 was observed in the epididymal tissue of obese animals. Although both the basal and stimulated release was found to be through a Golgi-dependant process, this release was transcriptionally regulated and, unlike leptin, IL-6 was not stored within the adipocytes prior to release.

The chronic exposure of differentiating preadipocytes to IL-6 enhanced lipid accumulation within the adipocytes. This lead to increased leptin secretion from the IL-6-treated cells, without an increase in *ob* expression. IL-6 did not affect the recruitment of preadipocytes to adipogenesis, nor did it increase the expression of the adipogenic modulators PPARγ or C/EBPα. However, in differentiated adipocytes, IL-6 treatment enhanced basal and insulin-stimulated glucose uptake and caused a reduction in adrenergically stimulated lipolysis.

In conclusion, chronic hyper-secretion of IL-6 from adipocytes causes increased lipid deposition and adipocyte hypertrophy, indirectly inducing insulin resistance.

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Abbreviations

ACRP30	Adipocyte complement-related protein of 30 kDa
ADD1SREBP	Adipocyte determination and differentiation-dependent factor 1/sterol regulatory element binding protein 1
ADRP	Adipose differentiation-related protein
BCS	Bovine calf serum
BMI	Body mass index
BSFp-2	B-cell stimulatory factor 2
C/EBP	CCAAT/enhancer binding protein
CCS	Cosmic calf serum
CNTF	Ciliary neurotrophic factor
COX2	Cyclooxygenase 2
CT-1	Cardiotrophin-1
DEPC	Di-ethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DOG	Deoxyglucose
EGF	Epidermal growth factor
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde-phosphate dehydrogenase
GAS2	Growth-arrest-associated gene 2
GLUT	Glucose transporter
HSL	Hormone-sensitive lipase
IBMX	Isobutylmethylxanthine
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL-6	Interleukin-6
ILRE	Interleukin response element
IR	Insulin receptor
IRS-1	Insulin-receptor substrate-1
JAK	Janus kinase
LIF	Leukaemia inhibitory factor
LPL	Lipoprotein lipase

MAPK	Mitogen-activated protein kinase
MRE	Multiple response element
NF-IL-6	Nuclear factor for interleukin-6 expression
NSAID	Non-steroidal anti-inflammatory drug
OSM	Oncostatin-M
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PG	Prostaglandin
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptors
rt-PCR	Reverse-transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
T_3	Tri-iodothyronine
TGF-β1	Transforming growth factor-β1
TNFα	Tumour necrosis factor α
TZD	Thiazolidinedione

Chapter 1 – Introduction and Literature Review

Obesity

Obesity is one of the most important health issues in the developed world, and its spread is extending further into the developing world. The current World Health Organisation definition of obesity is a body mass index (BMI) greater than 30 kg/m^2. A 2001 report by the National Audit Office in England estimated that 65% of English adults were overweight (a BMI greater than 25), with 19% of the population obese. In 1980 this figure was approximately 7% [1]. In the USA, an estimated 61.3 million people are obese: 27% of the population [2]. As a result, obesity commands the second highest cost in healthcare in these countries, after smoking-related diseases [3]. Numerous pathologies are associated with obesity [4], the most prominent being type 2 diabetes, insulin resistance and cardiovascular disease.

The majority of researchers have attributed the rise in obesity, particularly among children, to a combination of increased intake of inappropriate (high-fat, high-carbohydrate) food and a sedentary lifestyle [5]. Other influences are involved, particularly genetic susceptibility [6], which might affect weight gain, pathogenesis of disease and treatment. Notably, whether through genetic predisposition or lifestyle, certain ethnic groups appear to have a greater propensity towards obesity. Among the most studied are the native American Pima Indians [7], who have a higher proportion of insulin resistance and type-2 diabetes than Caucasian populations. Other ethnic groups appear to have a greater prevalence or propensity for obesity and its associated pathologies compared with Caucasians in similar communities [8-10]. Additionally, other theories have been advanced to explain the rise in obesity, including an adenovirus originally observed in chickens [11] and pollution [12].

Physiologically, obesity is characterised by an excess of adipose tissue. All adipose is not created equal, however, and can broadly be divided into two: subcutaneous adipose tissue, which is located between the muscle and skin, and can be found all over the body [13]; and visceral

(or omental) adipose tissue, which lies primarily in the body cavity, surrounding the internal organs. The depots differ for various biochemical functions, including adipogenesis [14, 15], lipolysis [16], glucose uptake [17], the release and/or expression of adipokines or other adipocyte factors [18, 19] and their response to stimuli [20]. Furthermore, the deposition of visceral fat (rather than subcutaneous) appears to be a greater risk factor for insulin resistance, type-2 diabetes and cardiovascular disease [21, 22].

Therefore, the study of adipose tissue and the understanding of the mechanisms involved in adipose tissue function will help to prevent and treat obesity and the diseases with which it is associated.

Adipose Tissue

Adipose tissue is composed of a variety of cell types, including adipocytes, preadipocytes, blood vessels, neurons and immune cells. Of note, especially concerning the adipocyte release of inflammatory cytokines, is the increased infiltration of macrophages into the adipose tissue in the obese state [23] and the reported conversion of adipocytes to macrophages [24]. The precise constitution of adipose tissue varies with depot and obesity, but it has been estimated that up to ~60% of cells in the tissue could be macrophages [23].

This section will introduce the anatomy and physiology of the adipocyte and adipose tissue, as well as its relevance to the organism and disease. A number of the most prominent adipokines will be reviewed, excluding interleukin-6 (IL-6), which is described in the subsequent section.

The adipocyte

The adipocyte from white adipose tissue has many roles (although humans, apart from neonates, do not have traditional brown adipose tissue as other mammals do, there is a degree of plasticity within human adipose tissue [25]. It should be assumed that all data reviewed and presented here are based upon white adipose tissue). It is, of course, a storage depot hoarding excess energy as triglyceride within lipid vacuoles in the cell, enabling energy release in times of insufficient energy input. As such, it also has a role in glucose metabolism and insulin sensitivity. Furthermore, as part of the adipose tissue it has other roles, namely insulation and protection for the internal organs. More recently, however, the importance of the adipocyte as an endocrine signalling cell has been recognised.

Adipocytes are formed from stem cells of the fibroblast lineage in response to various stimuli [26]. An adipocyte is clearly distinguishable from a preadipocyte by its rounded shape (whereas the preadipocyte tends to be spindle-shaped) and lipid vacuole. Numerous adipocyte-

specific genes, including the *ob* gene (the product of which is leptin) and adipsin are expressed [27], and other genes that are required for adipocyte function, such as the glucose transporter GLUT4 [28] or lipoprotein lipase (LPL) [29], are upregulated during differentiation. Once formed, the adipocyte is able to carry out its role in storage and signalling.

It is becoming apparent that obesity affects the normal function of the adipocyte. Hypertrophy, rather than hyperplasia, appears to be the main mechanism involved in non-morbid obesity [30, 31] (certain forms of obesity do appear to involve hyperplasia in addition to hypertrophy [32, 33]). This is significant, because hypertrophic adipocytes appear to have altered functions compared with cells containing less lipid. Leptin expression, for example, is greater in large adipocytes [34, 35].

Humans, animals and cell lines – how adipose tissue is studied

As with most biological systems, the physiology, anatomy and function of adipose tissue can be studied using one of three general methods: humans, animals (typically rodents) or cell lines. Although not necessarily specific to adipocyte research, the benefits and problems of each will be briefly discussed.

Study on humans is, of course, the gold standard. There are numerous types of study that can be carried out on volunteers, the least invasive of which tend to be the associative studies. These usually involve the correlation of various indices of obesity (BMI, percentage body fat as measured by bio-impedance, dual-energy x-ray absorptiometry (DEXA) or callipers, or waist-to-hip ratio) with particular molecules (often adipokines) in the circulation. More invasive procedures involve the cannulation of veins that drain specific adipose tissue depots. By comparing the concentrations of adipokines or other products in the plasma or serum from these locations with those in arterial samples (representing the circulating levels) an index of what is released or cleared by the adipose tissue can be created [36, 37]. Moreover, molecules of interest can be injected into humans and the effects on body fat and adipose tissue can be investigated using the methods above.

Furthermore, microdialysis techniques have been adapted for *in vivo* analysis and enable the continuous sampling of molecules and metabolites from the intracellular space surrounding subcutaneous adipose tissue [38]. Finally, adipose tissue from various depots can be excised and investigated *in vitro*. This enables expression or release studies of different tissues, the study of specific molecular processes in the adipocyte that would be difficult *in vivo* and the investigation of adipose tissue without the fear of toxicity or further injury to the subject. In addition to direct measurements from explanted tissue, human adipose tissue can be maintained in primary cultures for longer-term studies or exogenous-compound treatments that would be impractical *in vivo*. There are problems with humans as a study material: subjects must be volunteers, ethical considerations are far more stringent and social, genetic and background conditions are heterogeneous.

Animals (rats and mice are the most frequently used for research into obesity) are often an effective substitute for humans. They are cheap, pose less of an ethical problem and enable a wider scope for intervention. Furthermore, they are genetically homogeneous, and can be fed and exercised in regimented and controlled environments for the duration of their lives. Numerous strains of mouse and rat have contributed to our knowledge of obesity. As well as investigating the effect of specific diets, and how they cause obesity, transgenic animals lacking various genes have helped to identify products such as leptin and its receptor system. A non-exhaustive list of animals used includes $ob^{-/-}$ and $db^{-/-}$ mice, both of which suffer early-onset obesity (and its related pathologies) and were found to lack leptin and its receptor, respectively. Additionally, gene knockouts of tumour necrosis factor (TNF)-α and IL-6 have also been used, as well as knockouts for other intracellular adipocyte factors. These are in addition to the methods described for use in human studies, enabling animal models to be used as versatile materials for obesity research. Problems do exist, not least because humans and mice are very different. Apart from the obvious anatomical disparity, there are considerable molecular differences. This is well illustrated by the

discovery of resistin in murine obesity, a molecule believed to link obesity to insulin resistance [39] (see 'Other adipokines'), but for which a precise human homologue has yet to be identified.

The third method available to researchers is the use of cell lines. In addition to adipose tissue obtained from human volunteers or animals, from which the adipocytes can be isolated and grown, are preadipocyte cell lines. Most frequently used are the cell lines 3T3-L1 and 3T3-F442A. These, in addition to ob1771 cells, are fibroblasts that are committed to the adipocyte lineage. There are also uncommitted stem cells with the ability to differentiate into adipocytes, such as 3T3-NIH, 7F2 or CRL-12557 cells. Cell lines have the advantage of requiring no ethical approval and a population of identical cells that can be treated with defined media. 3T3-L1 cells, in particular, have provided considerable information on the molecular mechanisms of adipocyte function. Developed during the 1970s [40, 41], much of the work on adipocyte differentiation has been carried out using this cell line. However, the strengths of cell lines are also one of their weaknesses. Although using defined media is helpful in investigating specific processes, there is no guarantee that similar mechanisms exist *in vivo* or truly represent the physiological state, which cannot be recreated in these systems.

Therefore, it is worthwhile considering the source of information when investigating research on adipose tissue and obesity. Undeniably, however, the three forms of study have contributed massively to our understanding of the adipocyte and adipose tissue.

Adipogenesis

Adipogenesis is the terminal differentiation of a preadipocyte or stem cell into an adipocyte. For adipogenesis to occur, a number of requirements must be met, including growth arrest and the expression and activation of the transcription factors – proteins that can bind to promoter regions of DNA to enhance or reduce gene expression – that control adipogenesis. Most data concerning adipocyte differentiation have come from the cell line 3T3-L1, and latterly from knockout animals lacking various

transcription factors. This has resulted in detailed information pertaining to adipogenesis *in vitro*, but relatively little information as to the requirements *in vivo*. This section will describe the mechanism of adipogenesis, the methods used to achieve it *in vitro* and how they relate to the *in vivo* system.

Three major families of transcription factors are involved in the differentiation of adipocytes: CCAAT/enhancer binding proteins (C/EBPs), peroxisome proliferator activated receptors (PPARs) and adipocyte determination and differentiation-dependent factor 1/sterol regulatory element binding protein 1 (ADD1/SREBP1). Of the three families, ADD1/SREBP1 appears to have the least influence on adipogenesis. The ectopic expression of ADD1/SREBP1 is insufficient to initiate differentiation, although it does enhance the process under the correct conditions [42]. Instead, it seems probable that ADD1/SREBP1 controls the expression of some adipocyte genes, including LPL and fatty acid synthase (FAS) [42, 43], and is possibly involved in the synthesis of PPARγ ligands [44].

The central molecular basis of adipogenesis can be ascribed to four transcription factors, which are expressed within hours of exposure to adipogenic stimuli. These are C/EBPβ, C/EBPδ, C/EBPα and PPARγ (specifically PPARγ2). Broadly, C/EBPβ and C/EBPδ are rapidly transcribed and, together, result in the expression of C/EBPα and PPARγ, which are the most important transcription factors that are involved in adipocyte differentiation (Figure 1.1). C/EBPα and PPARγ maintain the expression of each other, and upregulate the transcription of most of the genes that are required for adipogenesis and the function of the mature adipocyte. The expression of C/EBPβ and C/EBPδ wane once PPARγ and C/EBPα have been induced [45, 46].

Members of the C/EBP family of transcription factors are bZIP proteins, containing a basic leucine zipper domain at the C-terminal for binding to DNA [47]. Following the adipogenic signal, the immediate and transient expression of C/EBPβ and C/EBPδ occurs [48]. Of the two,

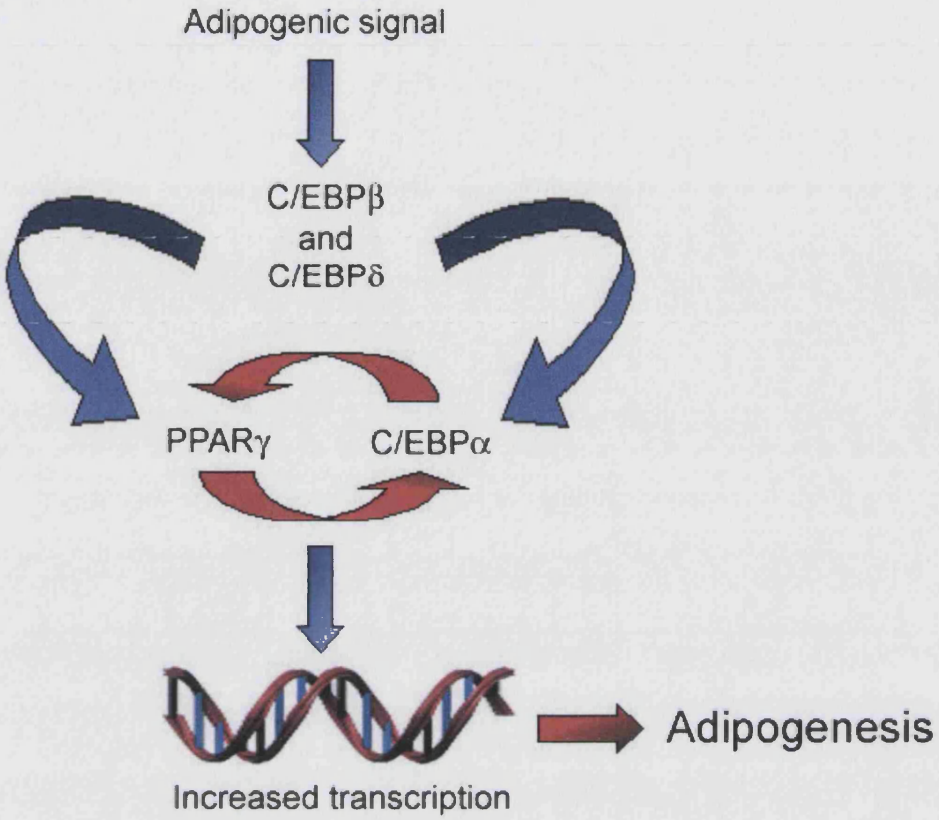

Figure 1.1 – Transcription factors involved in adipogenesis.

Adipogenic signals are initially mediated through the transcription factors C/EBPβ and C/EBPδ. These two factors increase the expression of the two major adipogenic transcription factors, PPARγ and C/EBPα, which are self-perpetuating and initiate the transcription of all the genes that are required for adipogenesis to occur.

C/EBPβ has the more fundamental role; the ectopic expression of C/EBPβ alone is sufficient to initiate adipogenesis in the absence of any external stimuli [49], whereas C/EBPδ expression is insufficient to achieve this.

The transience of expression of C/EBPβ and C/EBPδ is because their sole purpose appears to be to initiate the transcription of the central controlier of adipocyte differentiation, PPARγ [50, 51]. The third member of the C/EBP family that occurs in adipocytes, C/EBPα, is expressed concurrently with PPARγ. C/EBPα is a powerful inducer of adipogenesis, and ectopic expression is sufficient to induce differentiation in the absence of external factors [52]. C/EBPα controls the expression of a number of vital genes within the adipocyte; in addition to PPARγ, C/EBPα upregulates the transcription of adipsin, leptin, β3-adrenoceptors, insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) [53-55].

Undoubtedly the most important factor that is required for adipogenesis is PPARγ2, the adipocyte-specific isoform of PPARγ [56]. Although PPARγ1 is also expressed in adipocytes, the two isoforms differ at the amino-terminus, with PPARγ2 containing an extended 30-amino acid sequence [57]. PPARγ2 is approximately five times more effective at stimulating transcription in adipocytes than is PPARγ1 [58], and PPARγ2 (but not PPARγ1) expression alone can restore adipogenesis in PPARγ-deficient adipose tissue [59]. PPARγ exerts its effects through heterodimerisation with RXR, the retinoid-X receptor. Within the promoter region for PPARγ lies a C/EBP-binding consensus sequence, thus explaining the upregulation of PPARγ by the C/EBPs [50, 60]. However, PPARγ is such a potent stimulator of adipocyte differentiation that adipogenesis will occur even in the absence of any member of the C/EBP family [59, 61]. This is also true for other cell types, such as human liposarcoma [62] (the expression of both PPARγ and C/EBPα also results in adipocyte-like differentiation in other cell types [63]). Furthermore, PPARγ can restore considerable adipocyte function to C/EBPα-knockout

cells [54]. C/EBPα is inadequate for restoring adipogenesis in PPARγ-deficient cells [64], and PPARγ-deficient animals are frequently nonviable during gestation, with survivors having little adipose tissue [65]. As the central molecule in adipogenesis, therefore, the action of PPARγ is likely to result in the expression of almost all adipocyte genes that are not specifically controlled by C/EBPα or ADD1/SREBP1. It is also required for the continued expression of C/EBPα (and vice-versa) [66]. Antagonism between PPARγ and C/EBPα also exists for the expression of *ob*, in which the former inhibits expression and the latter increases it [53]. Whether this is a common phenomenon has yet to be established.

Promoters of adipogenesis

In vitro differentiation of adipocytes uses a number of different methods but all follow the same general principles: growth arrest must occur, PPARγ and C/EBPα must be induced and the anti-adipogenic effect of any other factors must be minimised. Although numerous compounds are available to induce differentiation, only one is central to all methods: insulin. Insulin, however, has no direct effect on adipogenesis. It does potentiate the effect of other factors [67], increasing the number of cells within the population that differentiate, as well as increasing lipid accumulation in the differentiated adipocytes [68]. Differentiation without lipid accumulation can occur [69] but this is rarely desirable. Insulin-like growth factor-1 (IGF-1) is also required, but *in vitro* this is a component of calf serum, and thus supplemented in the media [70, 71]. The thyroid hormone triiodothyronine (T_3) enhances insulin sensitivity and glucose uptake, and has been used in the differentiation of adipocytes [72].

Therefore, in addition to insulin, other mediators must be used. Glucocorticoids, such as dexamethasone, are potent effectors of adipocyte differentiation, with myriad pro-adipogenic effects. Dexamethasone causes a rapid rise in the expression of C/EBPδ [73, 74], possibly by the activation of the glucocorticoid receptor, which is a member of the PPAR superfamily. It also: downregulates the growth-

arrest-associated gene 2 (GAS2), enabling post-mitotic growth arrest [75]; reduces β-adrenoceptor density on the cell surface, reducing lipolysis [76]; increases intracellular cyclic AMP (cAMP) [77]; and downregulates *pref-1* (preadipocyte factor-1), a gene product that is responsible for maintaining the cell in the preadipocyte state [78]. Additionally, in association with insulin, it increases the expression of GLUT4, enabling the enhancement of glucose uptake and lipid deposition [79]. Another powerful inducer of adipogenesis is cAMP, the levels of which are increased in differentiating adipocytes by the use of the non-specific phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Raising cAMP levels within the cell has numerous effects, but those relevant to adipogenesis include the inhibition of hormone sensitive lipase (HSL) activity [77], the suppression of TNFα expression [80], increasing intracellular levels of C/EBPβ [81], and decreasing levels of the C/EBPα promoter repressor Sp1 [82]. IBMX is usually used in conjunction with dexamethasone and insulin.

Direct ligands for PPARγ are also excellent adipogenic factors. Natural ligands include a number of members of the prostaglandin (PG) family, which are produced by mature adipocytes [83]. $PGF_{2\alpha}$ is a potent stimulator of differentiation [84], although possibly through a non-PPARγ-related mechanism. However, 15-deoxy-Δ12,14-PGJ_2 can directly bind to and activate PPARγ, and might be an endogenous ligand for adipogenesis *in vivo* [26, 85]. Prostaglandins are rarely used *in vitro*, and are substituted for either indomethacin or members of the thiazolidinedione (TZD) family of compounds. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), binds to and activates PPARγ, resulting in differentiation [86, 87]. Other NSAIDs can induce adipocyte differentiation, notably ibuprofen, but are markedly less potent than indomethacin, implying that the mechanism is through the activation of PPARγ rather than through changes in cyclooxygenase activity [88]. Finally, TZDs are extremely effective modulators of PPARγ activity and adipogenesis [89], which has been suggested as the source of action for

their anti-diabetic properties. TZDs are potent enough to induce adipogenesis in cell lines that are not normally involved in the formation of adipocytes, such as stromal bone-marrow cells [90]. Indomethacin and TZDs are used to differentiate adipocytes *in vitro*, the former with insulin, and the latter in combination with dexamethasone, IBMX and insulin.

In spite of what is known about the modulators of adipocyte differentiation *in vitro*, relatively little is understood about endogenous activators *in vivo*. Prostaglandins are a possibility, as are T_3, insulin and insulin-like growth factor-1 (IGF-1). Other molecules that have been investigated, such as growth hormone, have successfully differentiated cell lines but inhibited adipogenesis in primary culture, making them unlikely *in vivo* effectors [91].

Inhibitors of adipogenesis

A number of endogenous inhibitors of adipocyte differentiation have been identified. Probably the best characterised inhibitor is TNFα, which not only prevents differentiation but reverses the commitment of cells that are already undergoing adipogenesis and induces apoptosis in mature adipocytes [92, 93]. Other cytokines and growth factors can also inhibit adipogenesis, in particular IL-1β and a variety of growth factors [94]. This inhibition appears to be by the activation of various MAPK (mitogen-activated protein kinases) intermediaries, which appear to bind to, phosphorylate and inactivate PPARγ2 [95].

One preadipocyte factor that is absent in mature adipocytes appears to be responsible for maintaining the cell in the undifferentiated state. Pref-1 is a transmembrane protein containing epidermal growth factor (EGF)-like repeats [96]. Its mechanism of action has not been described, but it appears only to function when released as a secreted factor, created by alternative splicing or proteolytic cleavage [97]. It, therefore, appears to be a novel autocrine/paracrine repressor of adipocyte differentiation. Pref-1 is not a good marker for preadipocytes, however, because although it inhibits adipogenesis, its presence is neither required for the preadipocyte state nor its absence a signal for

differentiation [98], and its expression is not restored by the addition of TNFα [99]. It is most likely to be a factor that prevents differentiation if present, and is completely absent in the mature adipocyte.

Adipose tissue as an endocrine organ

With the realisation that adipose tissue was secreting a considerable number of factors into the circulation, the tissue needed to be reclassified from an energy-storage cell to a multi-functional organ. The identification of the product of the *ob* gene, leptin, as an adipocyte-derived secretory signalling factor was the first step in this understanding [100, 101]. Subsequently, the production and release of TNFα and IL-6 were described [102, 103], and, more recently, resistin and adiponectin were discovered [39, 104, 105]. It is now widely accepted that adipose tissue is an endocrine organ [106, 107]. Of these candidates, only leptin and adiponectin are truly adipocyte derived; TNFα, IL-6 and resistin are (or a proportion is) released by the non-adipocyte fraction of the adipose tissue, of which infiltrated macrophages constitute a significant part [23] (although the functions of macrophage-like adipocytes have not been established [24]). Here, four of the major adipokines that are related to obesity (leptin, TNFα, resistin and adiponectin) will be discussed, in addition to IL-6.

Leptin

The search for leptin began with the development of a genetically obese mouse during the 1960s [108]. These $ob^{-/-}$ mice suffer from early-onset obesity as a result of severe hyperphagy [109], and had increased fat deposition compared with pair-fed wild-type counterparts [110]. The animals exhibit the pathologies that are associated with obesity, such as insulin resistance and hyperinsulinaemia [111]. The gene that is responsible for the obesity, the *ob* gene, was cloned in 1994 [100], and the product it encodes was isolated soon afterwards [101]. With the ability to reduce weight and feeding in $ob^{-/-}$ mice it was hailed as a satiety factor

that might cure obesity [112]. Although this was overoptimistic [113], the product of the *ob* gene, leptin, has revolutionised the study of adipose tissue biology.

Leptin was the first true endocrine molecule that was discovered to be produced largely by adipocytes. It is a 167 amino acid 16-kDa protein that is secreted from mature adipocytes but is absent from preadipocytes. Circulating concentrations or expression levels are increased in the obese and with age [114] and correlate with BMI [115], percentage body fat [116], total body lipid content [35] and adipocyte lipid volume [34]. The *ob* gene contains a binding site for the promoter C/EBPα, which appears to cause its transcription during adipogenesis [117]. Investigations using TZDs have shown that this C/EBPα effect is antagonised by PPARγ, with a dramatic reduction in leptin levels following PPARγ activation [53]. Expression levels of leptin vary considerably between adipose tissue depots, with subcutaneous adipose tissue release and mRNA levels considerably greater than those of visceral tissue [118]. Furthermore, the response to regulators of leptin expression or release, such as insulin, dexamethasone and TGF-β, was differentially modulated between these tissues [119-121]. Leptin signals through a system of leptin receptors, which are similar to gp130. A mutation in the leptin receptor is the fundamental defect of the diabetic $db^{-/-}$ mouse, which is obese and insulin resistant [122]. Leptin receptors are expressed in numerous peripheral tissues throughout the body, as well as in the brain and the central nervous system [123].

The biological effects of leptin are numerous, and some of its actions appear to be contradictory, especially in the case of autocrine/paracrine responses. Animal studies indicate a tendency to inhibit insulin action and lipogenesis and to stimulate lipolysis [124] (although possibly only in visceral depots [125]), and these effects have also been observed in human adipocytes [126]. Leptin has also been implicated in adipocyte apoptosis and the control of cell numbers [127]. Correlations between leptin and whole body insulin resistance have been observed, as well as the inhibition of insulin action on adipocytes [128,

129]. However, other studies have found no link between leptin and insulin resistance [130] or have identified leptin as an anti-diabetic agent [131]. Furthermore, lipodystrophic subjects, with little adipose tissue and low levels of leptin, suffer from severe insulin resistance that is reversed by the administration of leptin [132]. Part of this confusion might be a result of leptin resistance, with the response of leptin in the obese being muted because of this [133]. This resistance might also be one of the reasons why leptin was less successful as a satiety factor than was hoped. Leptin, under normal circumstances, is a potent satiety factor. The expression of *ob* is markedly reduced in fasting animals, and increases upon feeding [134, 135], and circulating leptin is able to cross the blood–brain barrier and act centrally to reduce feeding [136]. Administering leptin to humans has not been particularly successful in combating obesity [113], except in the case of individuals with congenital leptin deficiency [137]. One further significant metabolic effect of leptin is the apparent inhibition of insulin release from pancreatic β-cells [138]. The role of leptin in feeding, obesity and impaired insulin action is complex, made more difficult by the effects of insulin resistance, and despite the research effort thus far, no clear overall mechanism has been delineated.

In addition to these effects, leptin has many other actions: it is a permissive factor for the onset of puberty, in which it has been suggested that leptin acts as a signal of sufficient fat stores, enabling the process to begin [139]; it is a growth factor promoting haematopoiesis [140] and angiogenesis [141]; it causes the proliferation of pancreatic β-cells [142], embryonic cells [143] and pituitary cells [144]; it is involved in wound healing [145]; and it is a differentiation factor for osteoblasts [146].

Leptin, therefore, is a true adipocyte-derived hormone, and certainly the first reported to have central as well as peripheral effects. Whether it has autocrine/paracrine effects, and what they are, is more contentious. The expression of *ob* has recently been detected in other tissues, including keratinocytes, endothelial cells, pituitary cells and the stomach [145, 147, 148]. How this relates to adipocyte-derived leptin is unknown.

TNFα

As its name suggests, TNFα was originally described as a factor that induces necrosis in tumours, and is released from macrophages in response to endotoxin stimulation [149]. A second molecule was later described, which was termed cachectin and was also macrophage derived, which dramatically reduced the activity of lipoprotein lipase (LPL) in adipocytes [150], as well as reducing the expression of a number of adipocytes genes, including adipsin [151]. Cachectin was subsequently identified as TNFα [152]. TNFα is also involved in the acute phase response, and can induce IL-6 secretion [153]. More recently, its expression has been shown in adipocytes, where it might have an autocrine/paracrine role in metabolism [102].

TNFα is a 26-kDa transmembrane protein, which can be cleaved to create a soluble 17-kDa form of the protein, although both forms of the molecule are biologically active [154]. The actions of TNFα are dependent upon two receptors, TNFR1 and TNFR2 [155]. Both receptors can bind to TNFα and result in signal transduction, and it is possible that the varying actions of the cytokine are modulated by each receptor [156]. TNFR1, for example, has been implicated in cytotoxic and apoptotic signalling [157], whereas TNFR2 has been described as a ligand-passing receptor [158]. Despite this, both receptors have the ability to initiate phosphorylation cascades and activate multiple pathways [159].

The metabolic effects of TNFα have been well studied, especially concerning insulin resistance and diabetes [160]. Curiously, however, despite the fact that TNFα is expressed and produced by adipose tissue, and that this production is significantly increased in obese individuals, it does not appear to be released by adipose tissue or increased in the circulation [103, 161]. Nevertheless, TNFα remains a potent modulator of adipocyte function. As described by the actions of cachectin, it is a potent inhibitor of the actions and production of the lipogenic enzyme LPL, and causes an increase in lipolysis, contributing to hyperlipidaemia [162]. Furthermore, TNFα induces apoptosis in adipocytes [93, 163], particularly

from visceral adipose tissue [164], in addition to its ability to inhibit the differentiation of adipocytes through PPARγ [165]. It also increases the release of leptin from adipocytes without affecting transcription [166]. Finally, TNFα directly affects insulin signalling at numerous points within the pathway, therefore inhibiting its action. It suppresses the phosphorylation of the IR and its substrates to inhibit the activation of the signalling cascade [167], as well as reducing the expression of the insulin-dependent glucose transporter GLUT4 [168], which ultimately results in whole-body hyperglycaemia [169].

Of particular interest, in terms of both the treatment of disease and for research purposes, is the fact that all of these functions of TNFα are antagonised and reversed by the TZDs. The addition of TZDs *in vitro*, or the administration of the compounds *in vivo*, improves insulin sensitivity by increasing both the expression and phosphorylation of GLUT4 [170], enabling the differentiation of adipocytes [171] and inhibiting TNFα-mediated lipolysis [172]. The expression of TNFα in adipocytes does not appear to be directly affected by TZDs [173], but an improvement in metabolic status and weight loss following TZD treatment might reduce TNFα levels. It has been suggested that TNFα in adipose tissue is designed to prevent weight gain by limiting the availability and function of adipose tissue, but that this results in insulin resistance and the deposition of lipid with other tissues, such as muscle and liver [174].

Other adipokines

During the past three years, tremendous interest in two novel adipokines has been aroused. These two molecules, resistin and adiponectin, are adipose-specific cytokines that have been implicated in the development of insulin resistance. With only a few years' research, the information on both of the adipokines is limited and their relevance is as yet undetermined, but the possibilities (especially of adiponectin) are intriguing.

Resistin (which was briefly also known as Fizz3) was first identified in 2001, and levels of it were found to be increased in obesity. When levels of resistin were reduced by TZDs or anti-resistin antibodies, the accompanying insulin resistance was abolished [39]. It was also reported to inhibit adipocyte differentiation [175] and, working as a dimer [176], appeared to be the adipose-derived molecule that linked obesity and insulin resistance [177]. Just as quickly, however, contradictory reports were presented. Resistin expression was inhibited by both insulin and TNFα, which indicates that a role in insulin resistance is unlikely [178, 179]. Furthermore, resistin does not appear to be increased in all models of obesity [180]. The situation is complicated further by the fact that these findings were demonstrated in murine systems, and this has not translated well into humans. There are a number of significant differences between human and murine resistin, such that some have questioned whether, 'another as yet unidentified gene is the true human homolog of the murine *Retn* gene' [181]. Resistin expression in human adipocytes is minimal, with adipose tissue release accounted for by non-adipocyte cells, including preadipocytes [105]. Neither does it appear to correlate to indices of obesity, at least in cross-sectional studies [105], although correlations have been reported in longitudinal studies [182]. Resistin is expressed in other human tissues, however, notably macrophages and the placenta [183]. In macrophages, resistin expression is induced by cytokines, including IL-6, TNFα and IL-1β [184]. Whether this upregulation also occurs in adipose tissue is unknown.

Adiponectin, also known as ACRP30 (adipocyte complement-related protein of 30 kDa), was initially described in adipocytes, based upon its homology to the complement factor C1q [185]. It shares evolutionary homology with TNFα [186] and its release from adipocytes is enhanced by insulin [187]. Since 2001, promising data have been published concerning the effects of adiponectin on insulin sensitivity. In both hepatic tissue and muscle, incubation with adiponectin improves insulin action [188]. *In vivo* experiments have provided some particularly interesting results, because, unlike the majority of other adipokines,

adiponectin has a positive effect on insulin sensitivity. Not only does administering adiponectin to obese or diabetic organisms improve insulin action [189] but a lack of the adipokine results in insulin resistance [190]. Circulating levels of adiponectin are reduced in obese individuals, with plasma concentrations having a negative correlation with various indices of obesity and insulin resistance [191, 192], a situation that does not appear to be shared by any other major adipokines that correlate with obesity. The release of adiponectin is greater from visceral adipose tissue depots, and it appears that release from this depot correlates more conclusively with obesity [20]. There does not appear to be acute control of the expression or release of adiponectin [193], but the adipokine does appear to be under the control of the transcription factor PPARγ; treatment with TZDs increases adiponectin levels [194], and polymorphisms in the PPARγ gene affect circulating levels of the adipokine [195]. Whether the obesity-related reduction in adiponectin release is related to PPARγ effects remains to be determined.

Adiponectin appears to be an excellent target for links between obesity and its related metabolic diseases. The role of resistin is more controversial, but (as with adiponectin) too little is known to draw meaningful conclusions from either adipokine.

Interleukin-6

A brief history

The molecule that is now referred to as IL-6 was originally identified in 1987, under a number of different guises, by a number of research groups. It was initially reported in 1980 from the cloning of its mRNA as interferon-β_2 (IFN-β_2), owing to similarities in anti-viral activity with IFN-β, [196]. It was subsequently isolated and sequenced by a number of separate research groups (and known as 26K factor, on account of its size), noting that it had interferon-like activity but no apparent structural homology [197].

A growth factor for hybridomas and plastocytomas in mice was isolated from helper T cells and macrophages [198]. A similar human molecule was also discovered, and its sequencing revealed a homology not to the mouse protein, but to IFN-β_2/26K [199]. In separate experiments, a third molecule, B-cell stimulatory factor 2 (BSFp-2), was isolated based on the ability of a T-cell product to stimulate the release of immunoglobulin from B cells [200]. The cloning of this revealed it to be an interleukin, and to be identical to IFN-β_2/26K. Notably, this is not an exhaustive list of the names for IL-6, most of which were derived from its actions on a variety of cell types, but an indication of the methods that lead to the sequencing and positive identification. Once the sequence was published, other factors, such as hepatocyte-stimulating factor, T-cell replacing factor and thrombopoietin, were found to be identical to IL-6 [201, 202].

The structure of both the gene and the protein of IL-6 are now well characterised. At the DNA level, there is 65% homology between the mouse and human gene [203], and both genes have four introns and five exons. The proteins share 42% homology, although much of the divergence occurs at the amino terminus, which does not appear to contain vital functional information [204]. Human IL-6 is a 21–28-kDa

protein, depending upon post-translational modification by both N- and O-linked glycosylation and serine phosphorylation [205]. Initially produced as a 212-residue protein, a 28 amino acid hydrophobic region is cleaved to release the mature protein. The solution structure of the protein was completed in 1997, indicating that IL-6 has five α-helices, four of which form a four-helix bundle (in common with other cytokines, such as leukaemia inhibitory factor, LIF, and ciliary neurotrophic factor, CNTF), which are separated by loops of various lengths [206]. The murine form of the protein is 22–29 kDa, but is not N-glycosylated, and consists of a 211 amino acid translated peptide from which 24 residues are cleaved [207]. In spite of the differences in post-translational modification, human IL-6 is active in murine systems, and non-glycosylated forms of the protein are functionally active.

The IL-6 receptor system

IL-6 is a member of a superfamily of cytokines, the class 1 cytokine receptor family, based upon a common receptor system. Other members of the family include LIF, oncostatin-M (OSM), CNTF, cardiotrophin-1 (CT-1) and interleukin-11 (IL-11), and a degree of functional redundancy is exhibited between the members [208]. All members of the family signal through the transmembrane receptor gp130, which contains cytoplasmic signal transduction domains similar to those of other cytokine receptors [209]. Although gp130 does not bind to the cytokines directly, it interacts with specific cytokine receptors that do not contain innate signalling capacity. A schematic of the receptor binding complexes formed is shown in Figure 1.2. The product of the *ob* gene, leptin, also signals through a similar system. Instead of binding to gp130, however, the leptin receptor (OB-R) is homologous to gp130 and contains its own signal transducing properties [210].

The IL-6 receptor (IL-6R) binds to IL-6 as a heterotrimer with two gp130 molecules, and is a 468 amino acid, 80-kDa peptide [211]. It has no intrinsic signalling domains, lacks tyrosine phosphorylation sites and is functional when the cytoplasmic region of the receptor is deleted,

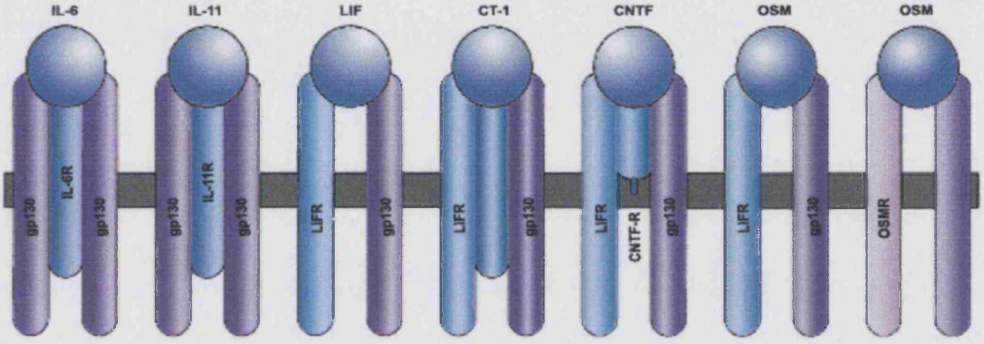

Figure 1.2 – Class 1 cytokine receptor binding to gp130.

Ligand and receptor binding to gp130 for signal transduction. Figure adapted from Heinrich *et al.* [217].

indicating that the purpose of the receptor is to bind to IL-6 and associate with gp130 [212]. In addition to its signal transducing function, gp130 also appears to create high-affinity IL-6 binding sites in conjunction with IL-6R, despite gp130 not directly binding to IL-6. The IL-6R exists in two forms, a membrane-bound receptor and a circulating soluble form (sIL-6R); the latter is formed by the proteolytic cleavage of the membrane receptor [213, 214]. Significantly, the binding of sIL-6R to IL-6 potentiates the activity of IL-6, enabling it to bind and activate gp130 without the involvement of the membrane-bound receptor.

None of the other members of the class I cytokine receptor family members binds to IL-6R, although a similar mechanism is used for each. LIF, CT-1, CNTF and OSM all bind to the LIF receptor (LIFR) and subsequently form a heterodimer with gp130, with the exception of CNTF, which also binds to its own receptor and forms a heterotrimer. OSM can also bind to a dedicated receptor, OSM-R, and subsequently bind to gp130 as a heterodimer. IL-11 is similar to IL-6, in that it binds to its own receptor (IL-11R) along with two molecules of gp130 (Figure 1.2) [215]. As does IL-6R, the leptin receptor exists in both soluble and membrane bound forms, termed short and long [216]. The short form is not only able to bind leptin in the circulation but also contains signal transducing capacity [217]. The soluble form of OB-R is formed by different mechanisms, depending upon the organism; soluble mouse OB-R is the product of a splice variant following gene transcription, whereas in humans, the formation mirrors that of sIL-6R, with proteolytic cleavage of the membrane receptor [218].

JAK/STAT and intracellular signalling

The activation of gp130 signal transduction by IL-6 binding results in an intracellular signalling cascade and the activation of nuclear transcription factors. Two major pathways are activated, the JAK/STAT (Janus kinase/ signal transducer and activator of transcription) pathway and the MAPK pathway. The ultimate targets for these cascades are the transcription factors STAT3 (STAT1 is also a minor IL-6 target) and nuclear factor

(NF)-IL-6, from the JAK/STAT and MAPK pathways, respectively. A representation of these two pathways is shown in Figure 1.3.

The JAK/STAT pathway involves the phosphorylation and activation of the JAK family of tyrosine kinases, followed by tyrosine phosphorylation of STAT3 and STAT1. JAKs are associated with the gp130 receptors at the cytoplasmic membrane region, prior to IL-6–IL-6R binding. IL-6 and its family members affect three Janus kinases: JAK1, JAK2 and Tyk2 [219]. Following the binding of IL-6–IL-6R to gp130, the receptor associated JAKs are brought into close association and become activated by tyrosine phosphorylation [219]. Activated JAKs phosphorylate tyrosine residues on the cytoplasmic region of gp130, providing a docking location for STATs. Once associated with gp130, the STAT is tyrosine phosphorylated by the proximal JAK [220]. Activated (phosphorylated) STATs dissociate from gp130 and dimerise, and transport to the nucleus (via diffusion), enabling DNA binding and the regulation of transcription [221].

The three JAKs that respond to IL-6 (and other members of the IL-6 family of cytokines), JAK1, JAK2 and Tyk2, are not equally potent. JAK1 appears to be the dominant molecule. Neither JAK2 nor Tyk2 could substitute for normal JAK1 functions in cells lacking this kinase [222]. Likewise, STAT3 is the major transcription factor involved in IL-6 signalling, acting as a STAT3 homodimer [223]. Whereas JAK1-deficient cells are unable to process IL-6 signalling, a deficiency in STAT3 alters the response, resulting in STAT1 homodimers and eliciting a response more akin to that of interferon-γ (IFN-γ) [221]. Under more normal conditions, STAT1 homodimers can be (but infrequently are) formed, as can STAT3–STAT1 heterodimers.

The alternative method for IL-6 signal transduction is via the MAPK pathway. The precise mediators of this cascade have not been identified, but the initial interaction between activated gp130 is with SHP2 (SH2-domain-containing tyrosine phosphatase). Phosphorylated (and therefore active) SHP2 can bind to and activate the Grb/SOS (growth factor receptor bound protein/son of sevenless) complex, subsequently binding

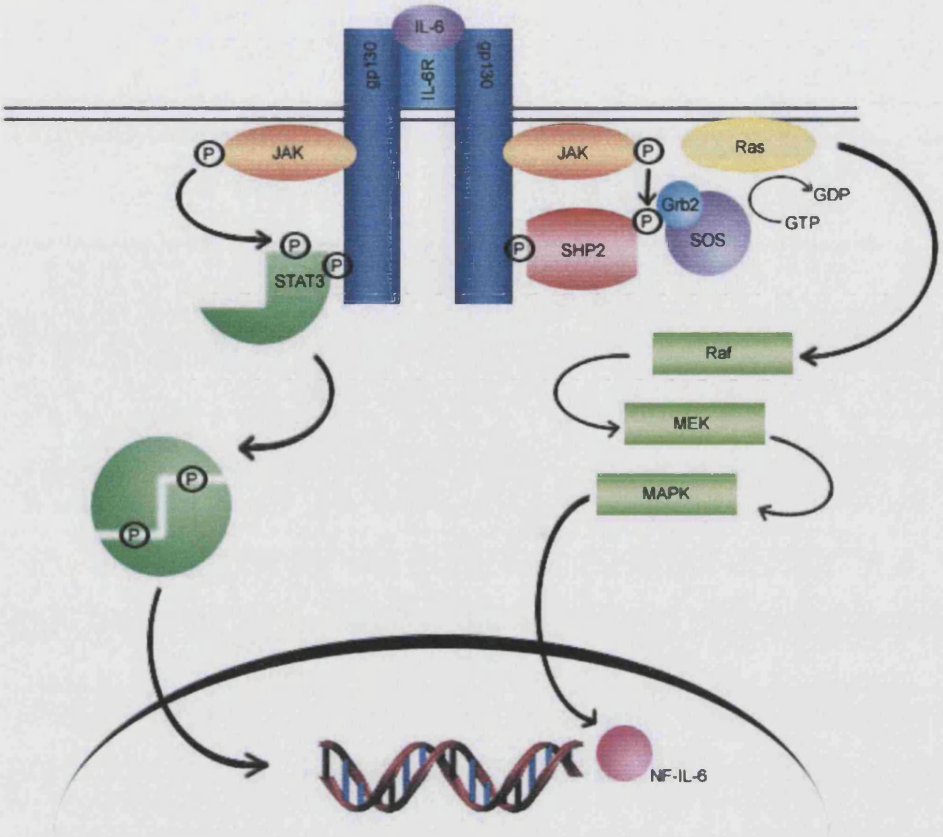

Figure 1.3 – IL-6 signalling through gp130.

The activation of gp130 by the binding of IL-6 to IL-6R results in the phosphorylation of gp130-associated JAKs. The subsequent tyrosine phosphorylation of gp130 enables the binding of intracellular messengers. The left-hand portion shows a simplification of the activation and dimerisation of STAT3, which diffuses to the nucleus and modulates transcription. The right-hand side shows the activation of the MAPK pathway; the activation of SHP2 and a signalling cascade involving the phosphorylation of a number of kinases. MAPK translocates to the nucleus and activates the transcription factor NF-IL-6 (C/EBPβ).

to Ras. This activates the MAPK pathway, involving the consecutive phosphorylation and activation of Raf, MEK and MAPK prior to the activation of the transcription factor NF-IL6 [224]. Intriguingly, NF-IL6 is also known as the transcription factor C/EBPβ, a member of the transcription family that is crucial for the differentiation of adipocytes [225]. In addition to activating the MAPK pathway, a complex involving SHP2 might also activate, or assist in activating, STATs. Notably, the small GTPase Rac-1, a member of the Raf family of signal transducing components, is involved in IL-6-induced STAT3 activation [226].

How these two pathways interact is still under investigation, leaving a number of questions unanswered. How does IL-6 elicit its multifarious responses using only two (major) transcription factors? How do the same factors elicit different responses in numerous cell types, following the same initial signal? And, with the (partial) redundancy that occurs among the cytokines, how are the specific signals from individual cytokines separated? Furthermore, it is not just IL-6 and the cytokines that signal via these intracellular messengers. MAPK signals can be initiated by stress signals, hormones, growth factors and other cytokines [227]. Mutational analyses of tyrosine residues in gp130 have indicated that JAKs, STATs and SHP2 bind to different moieties on the receptor [215, 228], but how the differential activation of each occurs is unclear. Other epitopes on gp130 differentially respond to the binding of the various members of the IL-6 family of cytokines [229], and intracellular adaptor proteins have been implicated in responses to specific cytokines [230]. Furthermore, IL-6 can induce contradictory signals in the same cell at the same time [231], indicating that the final response might depend upon other factors within the cell at the time of stimulation. Intracellular signalling is still poorly understood; although the major pathways have been elucidated, very little is known about specific control mechanisms or redundancy and promiscuity among the signal transducing elements.

Cellular sources and the release of IL-6

As indicated by its multiple discovery, IL-6 is released from a wide variety of cells [202, 232], including monocytes and macrophages, T cells, mast cells, fibroblasts, tumour cells, endothelial cells, epithelial cells, keratinocytes, skeletal muscle [232] and, of course, adipose tissue [103]. The regulation of IL-6 release differs among the cell types, even following the same stimulus. Lipopolysaccharide (LPS) induces IL-6 release from various cells, including macrophages and human umbilical vein endothelial cells (HUVEC) [198, 233], as do viral infections and other cytokines, such as TNFα and IL-1β [234]. IL-1β induces IL-6 release from adipocytes [235], and circulating levels of IL-6 are chronically raised in obesity, with significantly more release from omental adipose tissue depots [236].

The gene encoding IL-6, which is located on chromosome 7p21 [237], contains a number of transcriptional regulators, including a c-fos serum response element [203], a second messenger multiple response element (MRE) region and TATA box [238], an interleukin response element (ILRE) [239] and a C/EBP site [240]. These enable acute and specific control through a number of mediators. Nuclear factors that are known to activate the IL-6 gene include NF-IL-6 (C/EBPβ) and NF-κB, and repression has been shown by glucocorticoids. Polymorphisms in the (human) IL-6 gene that affect expression have also been observed, most markedly the –174 G/C polymorphism [241] which might be related to differences in insulin signalling.

The control of IL-6 release is, therefore, carefully regulated and dependent on a wide range of inhibitors and promoters in a number of cell types. Normal circulating IL-6 levels in humans are less than 3 pg/ml. Infection or injury can drastically, but acutely, increase these levels, up to levels greater than 200 pg/ml. Similar acute elevations are seen after exercise. Plasma levels tend to return to baseline rapidly (< 24 h) once the stimulus is removed. However, chronic elevation of IL-6 also occurs, notably in ageing [242], obesity [243], diabetes [244] and coronary heart

disease (CHD) [245]. Concentrations in these conditions rarely rise above 10 pg/ml, but levels remain elevated, and might be involved in pathogenic responses [246].

The acute phase response and other effects

Just as several modulators control IL-6 release, the protein itself has multifarious effects, again on a whole host of cell and tissue types. Obviously, many of these functions are those that led to the isolation and cloning of IL-6 itself: hybridoma and plasmacytoma growth, T-cell activation and B-cell differentiation, as well as an abundance of less well-studied roles. Possibly the best-documented role, however, is that of the acute phase response.

The acute phase response is the defence mechanism that is triggered following infection or injury in the organism. The pathway involves the local release of inflammatory mediators, including IL-6, as well as IL-1β, TNFα and interferons, followed by systemic reactions resulting in fever, lymphocyte proliferation and the release of acute phase proteins from the liver. Partial redundancy occurs among the cytokines in their ability to initiate the response, and the addition of one of the cytokines will (at least partially) cause the reaction to proceed [247]. However, although TNFα and IL-1β elicit most of the normal response, only IL-6 is capable of stimulating the release of all of the acute phase proteins [248]. Studies on human hepatocytes or cultured hepatic cells revealed the range of proteins that are upregulated by IL-6, including C-reactive protein (CRP), fibrinogen, serum amyloid A and haptoglobin [249]. As implied by its name, the reaction is acute; IL-6 is rapidly released, within 30 minutes of LPS injection, peaking at 2 h [250]. IL-6 clearance is also rapid, with a biphasic clearance consisting of an initial half-life of three minutes and a longer secondary half-life of 55 minutes [251].

IL-6 can also act as a growth and differentiation factor for a number of cell types. It is a potent activator of growth for both B-cell hybridomas and murine plasmacytomas [202], in which it can replace

feeder cells. IL-6 is required for terminal B-cell differentiation, without affecting resting B-cell status or B-cell growth [202]. T-cell activation by IL-6 occurs in synergy with IL-1β, resulting in thymic and peripheral T-cell proliferation and cytolytic T-cell responses [252].

This demonstrates the numerous actions of IL-6 as an acute activator of various facets of the immune system. The responses that are elicited are beneficial in fighting infection or repairing injury but would be deleterious to health if prolonged. Latterly, metabolic and endocrine effects of IL-6 have been described (of which some, but not all, are related to the acute phase response). The chronic elevation of plasma IL-6 in obesity, diabetes and cardiovascular disease has pathogenic consequences, which might, in part, be due to the inappropriate activation of the acute phase response, albeit at reduced levels. The metabolic and endocrine effects of IL-6, and the differences in chronic exposure, are discussed below.

Most of our knowledge of IL-6 comes from investigation into its immunological functions. Nevertheless, recent studies have indicated the importance of cytokines such as IL-6 in metabolic activity, not least the fact that IL-6 is released in significant concentrations by adipose tissue, especially in the obese [103]. Prior to this discovery, other 'inflammatory' cytokines, such as TNFα, had been reported in adipocytes, and implicated in pathologies of obesity [102]. The question still remains as to whether IL-6 has similar properties.

IL-6 and adipose tissue

In spite of the volume of literature that is available concerning the role of IL-6 and the immune response, relatively little is known about the role and regulation of the cytokine in adipose tissue, even compared with the other adipokines. It is released by adipose tissue [103, 253], and circulating levels correlate with all measurements of obesity and insulin resistance [254]. Its circulating levels are reduced with weight loss and reduction in fat mass [255], and its release, in obese subjects at least, is significantly greater from visceral adipose tissue [236]. Several factors have been reported to affect adipocyte release of IL-6: it is upregulated by β-adrenoceptor stimulation [256], catecholamines and insulin [257], IL-1β [235], exercise [258], TNFα and growth hormone, as well as IL-6 itself [259, 260]; and it is unaffected by food intake [261] or glucocorticoids [262] (although dexamethasone has been reported to reduce IL-6 expression [257]). This indicates that adipose-tissue-derived IL-6 is an autocrine/paracrine modulator, or acts on peripheral organs as a true endocrine signal. However, much of the research concerning the IL-6 effect on adipocytes is contradictory.

Notwithstanding the correlations with insulin resistance, IL-6 has been reported to increase basal and insulin stimulated glucose uptake in cultured adipocytes [263], or to have no effect [264]. Recently, however, it has been suggested that IL-6 reduces the expression of the insulin signalling components IRS-1 and GLUT4, as well as PPARγ [265, 266]. Similarly, there is little agreement concerning the effects of lipolysis. In breast adipocytes, IL-6 increased basal and stimulated lipolysis [267], and exercise-induced-IL-6 might also enhance the release of fatty acids from adipocytes [258]. Whole-body lipolysis increases with the infusion of IL-6 [268] and LPL activity is reduced [269]. However, other research has suggested that IL-6 has no effect on lipolysis in adipocytes [270]. The fact that IL-6 resulted in lipolysis from adipocytes surrounding lymph nodes but not gonadal adipocytes indicates that depot-specific differences in

response to IL-6 might occur [271]. IL-6 might also affect the expression or release of other adipokines. It has been reported to reduce the expression of leptin [272] and adiponectin [273] but to increase resistin levels [184].

Intriguingly, whole-body IL-6 knockouts are prone to late-onset obesity, which can be reversed by the administration of IL-6 [274], and intracerebroventricular IL-6 reduces food intake and body weight [275]. This suggests that IL-6, similar to leptin, has a central role in the development of obesity. Whether this translates into humans has yet to be established, although low levels of IL-6 in cerebrospinal fluid are inversely correlated with obesity [276]. The origin of cerebrospinal IL-6 is unknown, as is the ability of circulating IL-6 to be transported from the circulation.

Research into IL-6 and adipose tissue to date makes it very difficult to assess its importance. Although many statements have been made regarding its effects, disparity in the methods used ensures that different reports cannot be judged against each other adequately.

Aims of the study

Although the immunological effects of IL-6 have been well characterised, little is known of the importance of adipose-tissue-derived IL-6. Seemingly opposite effects have been recorded. This study, therefore, intends to investigate both the release and autocrine/paracrine effects of IL-6 in relation to adipose tissue.

First, the mechanism of IL-6 release from adipose tissue will be established. This will include investigations into the release of IL-6 from adipose tissue obtained from both lean and obese animals, to establish whether there is a fundamental difference in the tissue itself in the obese state. Furthermore, the variation in IL-6 release between visceral and subcutaneous depots will be examined.

The second part of this study will investigate the consequences of incubating IL-6 with cultured adipocytes, mimicking the autocrine/paracrine effects. Three major functions of adipocytes and adipose tissue will be examined: adipogenesis, glucose uptake and lipolysis. In addition, the effect of acute versus chronic incubation of IL-6 will be assessed.

Chapter 2 – Materials and Methods

Materials

Unless otherwise stated, laboratory chemicals and reagents were purchased from Sigma, Poole, UK.

Cell culture and laboratory plastics were purchased either from Corning Costar, Schiphol-Rijk, The Netherlands; Nalge–Nunc (via Fisher scientific, Leicester, UK); or Sarstedt, Leicester, UK. Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin and trypsin were obtained from Gibco (Invitrogen), Paisley, UK. Bovine calf serum (BCS) and cosmic calf serum (CCS) were from Perbio, Tattenhall, UK. 'Cell-gro' serum-free media (containing albumin) was supplied by Mediatech, VA, USA. β-adrenoceptor agonists were from Sigma, except CL316243, which was a gift from Gokhan Hotamisligil, Harvard University, USA. Differentiation agents were also supplied by Sigma, with the exception of BRL 49653 (rosiglitazone), which was a kind gift from GlaxoSmithKline, Harlow, UK. ^{3}H-deoxyglucose was purchased from NEN, MA, USA, and was measured using a Wallac (Turku, Finland) 1209 RackBeta liquid scintillation counter. Recombinant mouse and human IL-6 and mouse IL-1β were purchased from R&D Systems, Abingdon, UK.

Murine IL-6 and leptin ELISA kits were supplied by R&D Systems. Reagents for glycerol assay were from Sigma. Colourimetric assays were read on an Opsys MR (Dynex Technologies, Virginia, USA) plate reader, or a Uvikon 922 spectrophotometer (Kontron Instruments, Watford, UK).

Products for RNA isolation were all obtained from Sigma. Materials for cDNA synthesis were from Promega, Southampton, UK, except for nuclease-free water, which was supplied by Ambion, Huntingdon, UK. End-point reverse-transcription (rt)-PCR analysis products were also supplied by Promega. SYBR-Green reagents for real-time rt-PCR analysis were purchased from Applied Biosystems, Warrington, UK. All oligonucleotides were synthesised by Oswel, Southampton, UK. End-point rt-PCR was carried out on a Hybaid Omn-E thermal cycler (Thermo Hybaid, Ashford, UK): real-time rt-PCR on an ABI-PRISM 7000 (Applied

Biosystems). Agarose gel equipment was purchased from Bio-Rad, Hemel Hempstead, UK.

Probes for northern blot analysis were a generous gift from Gokhan Hotamisligil. Isolation material for these probes was purchased from Qiagen, Crawley, UK. Transfer paper was purchased from ICN, CA, USA or Amersham Pharmacia, Chalfont St. Giles, UK. Restriction enzymes were supplied by Promega. A Hoefer UVC 500 cross-linking machine was used, and blots were developed using a Kodak X-OMAT 1000 processor (Hemel Hempstead, UK).

Equipment for western blotting and reagents for protein estimation were purchased from Bio-Rad. Protease inhibitors ('Complete Mini' cocktail) were from Roche Diagnostics, Basel, Switzerland. ECL reagents were obtained from Amersham Pharmacia. Antibodies were purchased from Chemicon (Hampshire, UK) or Santa Cruz (CA, USA).

Animals were purchased from Harlan Sera-Lab, Loughborough, UK. The murine preadipocyte cell line 3T3-L1 was obtained from ATCC (VA, USA), and the 3T3-F442A cells were a gift from Gokhan Hotamisligil.

Methods

Preadipocyte cell culture

3T3-L1 and 3T3-F442A preadipocytes were grown on 75 cm^2 tissue culture flasks in 10 ml of DMEM containing 10% BCS and 1% penicillin/streptomycin, in a controlled environment at 37°C and 10% CO_2. Upon attaining near-confluence (approximately 85%), cells were trypsinised and passaged or seeded for differentiation. These cell lines were chosen for a number of reasons: 3T3-L1 cells are readily available from commercial sources; both cell lines are well established murine cell lines for adipocyte research [40], with numerous published methodologies [277, 278]; and the ability to differentiate these cells and use them for measurements was already established in our laboratory (unpublished data).

Cells for differentiation and experimentation were seeded onto 6-well tissue-culture plates at a density of 6×10^4 cells/well, and incubated at 37°C and 10% CO_2 in 1 ml DMEM/BCS. After 24 h, the media was changed to a new mixture of DMEM containing 10% CCS and 1% penicillin/streptomycin. Preadipocytes were grown to confluence at 37°C and 10% CO_2, whereupon they were transferred to a differentiation-induction medium.

Adipocyte differentiation

Upon attaining confluence, preadipocytes were treated for 72 h with a differentiation induction media designed to cause growth arrest, entry into the adipogenic cell cycle and lipid deposition [41, 279]. Unless otherwise stated, the induction media consisted of DMEM containing 10% CCS supplemented with the glucocorticoid dexamethasone (0.1 μM), the phosphodiesterase inhibitor IBMX (0.5 mM), insulin (5 μg/ml) and the thiazolidinedione BRL 49653 (rosiglitazone, 1 μM). These inducers (with or without thiazolidinediones) are the most frequently cited for the

differentiation of 3T3-L1 and 3T3-F442A adipocytes [280]. Although the precise concentrations of the compounds vary, they remain relatively similar (for example, dexamethasone concentrations are generally cited in the range 0.1–0.6 μM: IBMX is invariably used at 0.5 mM) [98, 166, 281]. Moreover, this technique was established within the laboratory (unpublished data). After 72 h incubation at 37°C and 10% CO_2, dexamethasone, IBMX and BRL 49653 were withdrawn and the cells fed with fresh DMEM/CCS containing 1 μM insulin every 48 h until > 90% of cells were lipid containing adipocytes (assessed by light microscopy).

Other adipogenic factors or methods were also used to assess adipogenesis (Chapter 4). The first alternative method substituted 1 mM IBMX for dexamethasone, BRL 49653 and 0.5 mM IBMX (dexamethasone was removed to investigate whether its numerous actions were essential for differentiation. One idea, which was not followed up owing to time limitations, was to investigate a more physiological method for differentiating adipocytes: the removal of dexamethasone was intended to be a starting point for this); the second substituted 125 μM indomethacin for dexamethasone, IBMX and BRL 49653. These cells were otherwise treated identically to the general method described above, with the 72 h incubation in the adipogenic mixture followed by feeding with DMEM/CCS supplemented with insulin every 48 h until fully differentiated. As with the dexamethasone, IBMX and insulin treatment, this is a well-established protocol [282]. A third alternative method involved feeding with DMEM/CCS supplemented with 2 nM T_3 and 17 nM insulin, in the absence of all other adipogenic factors, for both the initial 72 h and at every subsequent 48 h feeding [283, 284]. The precise methodologies are further described in Chapter 4, and their background is explored in Chapter 1.

Light and confocal microscopy

Cell confluence and differentiation were assessed by eye using light microscopy. To obtain a quantitative measure of differentiation (as defined by lipid accumulation), samples of cells were treated with the

fluorescent neutral-lipid-staining dye BODIPY 493/503 [285]. Adipocytes at selected time points during differentiation were displaced and seeded onto sterile coverslips. Cells were incubated for 15 minutes in a 40 μM solution of BODIPY 493/503 in HEPES buffer, pH 7.4. Cells were viewed on a Zeiss confocal microscope, with the dye excited at 493 nm and fluorescence measured at 503 nm. Images were viewed using LSM Image 5 Processor.

The BODIPY 493/503 images were then binarised above a threshold, so that all pixels containing signal (lipid droplets) were set to unity and all background to zero. A projection of a z-stack was used to include the full cell volume. The mean signal per cell was then expressed as a factorial value; total lipid engorgement could give a signal of one and no lipid a signal of zero. Quantitative measurements of BODIPY 493/503 were made using the processing package Lucidia (Kinetic Imaging, NC, USA).

Glucose uptake

3T3-L1 and 3T3-F442A cells were differentiated as described above, using dexamethasone, IBMX, insulin and BRL 49653. Once fully differentiated (assessed by microscopy) cells were treated for 24–72 h in Cell-gro, in the absence or presence of 10 ng/ml IL-6. Media was replaced every 24 h, where necessary. Cells were then washed with Krebs Ringer phosphate buffer, pH 7.4 (KRP: 128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$ and 1 mM Na_2HPO_4, at pH 7.4) warmed to 37°C, and incubated for 30 minutes at 37°C with Cell-gro, in the presence or absence of 2 μg/ml insulin. Ten minutes prior to the end of this incubation, 1 μl tritiated deoxyglucose (^{3}H-DOG) per ml media was added to each mixture. Following the incubation, cells were placed on ice and washed twice with ice-cold KRP buffer. Adipocytes were lysed with 0.1% SDS by incubating for 30 minutes at 37°C and homogenised by repeat pipetting. 800 μl of the homogenate was added to 8 ml scintillation fluid and the internalised ^{3}H-DOG measured by scintillation counting [171, 263].

A modified version of this protocol was used to measure protein levels by western blotting. No ^{3}H-DOG was used and instead of being lysed with 0.1% SDS, the cells were immediately flash frozen in liquid nitrogen and stored at –80°C. Protein levels were measured as described below.

Further details on these methods are described in Chapter 5.

Lipolysis

3T3-F442A or 3T3-L1 adipocytes were differentiated as described above, using dexamethasone, IBMX and insulin until > 90% of cells were lipid-containing adipocytes (as assessed by light microscopy). Adipocytes were then incubated at 37°C and 10% CO_2 for 8–72 h in Cell-gro, in the absence or presence of 10 ng/ml IL-6, re-feeding every 24 h, as required. Following this pre-incubation, cells were incubated (37°C, 10% CO_2) for 6 h in Cell-gro containing 1 μM dobutamine, 1 μM clenbuterol, 1 μM CL316243, 1μM noradrenaline, 2.5 mM dbcAMP or with no additive. The adipocytes that were pre-incubated with IL-6 were also incubated with 10 ng/ml IL-6 during the 6 h lipolysis incubation. After 6 h, the media was removed from the cells and assayed for glycerol accumulation [286]. The adipocytes were stored for RNA isolation. Further details are described in Chapter 5.

Animal explants

C57BL6/J or *ob*$^{-/-}$ mice were housed, fed, maintained and killed by Biological Services, UCL, according to Government guidelines. Weight measurements were made on live mice and blood collected by tail bleed. Subcutaneous or epididymal adipose tissue (the latter representing visceral or omental fat), or liver, was removed from recently killed animals, and washed twice in phosphate-buffered saline (PBS). Obvious blood vessels were avoided; however, adipose tissue contains a significant fraction of non-adipocyte cells, notably macrophages and stromal-vascular cells, the former of which increases with obesity (in which up to 60% of cells could be macrophages) [23]. The experiments

reported here do not differentiate between the release of IL-6 from the individual cell sources (the intention was also to study isolated adipocytes [235], but time constraints meant that this was not possible). Tissues were cut into small fragments and weighed prior to experimentation. Tissue fragments were incubated for 5 h or 24 h in Cell-Gro, containing 5 μg/ml IL-1β, 1 μM CL316243, 100 μg/ml cycloheximide or 0.5 μg/ml brefeldin A [166, 287, 288]. Following incubation, media was removed and retained for assay, and tissue was flash frozen in liquid nitrogen and stored at –80°C for RNA isolation. See Chapter 3 for further detail.

RNA isolation

To preserve the labile RNA, all products used for RNA isolation were treated with di-ethyl pyrocarbonate (DEPC). Water was sterilised by adding 0.01% DEPC, incubating overnight at 45°C and autoclaving. Chloroform, isopropanol and ethanol were prepared by adding 1% DEPC to each solution [289].

RNA was isolated from cultured cells using Tri-reagent and a phenol/chloroform method [290]. To each well of a 6-well tissue-culture plate 400 μl of Tri-reagent was added, mixed and incubated at room temperature for five minutes. Cells were homogenised by repeated pipetting and equivalent cells were pooled (where necessary). Samples were centrifuged at 12000 x g for ten minutes at 4°C. The supernatant solution was transferred to a fresh centrifuge tube and DEPC–chloroform was added (200 μl chloroform per 1 ml Tri-reagent used to digest the cells). Samples were mixed by vortexing and incubated at 4°C for 15 minutes. Samples were then centrifuged at 12000 x g for 15 minutes at 4°C and the supernatant solution removed. A $^{1}/_{10}$ volume of DEPC–isopropanol was added, vortexed and incubated at room temperature for two minutes, prior to centrifugation at 12000 x g for ten minutes at 4°C. The supernatant solution was again removed to a fresh tube and DEPC–isopropanol was added (500 μl isopropanol per 1 ml Tri-reagent used to digest the cells). Samples were incubated overnight at –20°C, and

subsequently centrifuged at 14000 x g for 30 minutes at 4°C. All liquid was removed and the pellet was washed in 1 ml (per 1 ml of Tri-reagent used to digest the cells) 75% DEPC–ethanol. Following further centrifugation at 14000 x g for 10 minutes at 4°C, the pellet was air-dried and reconstituted in 50 µl nuclease-free water.

Animal tissue RNA was extracted using 1 ml TRI reagent per (approximately) 500 mg tissue. Frozen tissue was crushed under liquid nitrogen to a fine powder and added to TRI reagent for homogenisation. It was subsequently isolated as described above.

cDNA synthesis

The concentration and purity of isolated RNA was assessed by measuring optical density at 260 nm (OD_{260}) and 280 nm (OD_{280}) [289]. A dilution of the sample of 4 µl in 1 ml DEPC-water was made in a crystal cuvette and measured at both OD_{260} and OD_{280} (using pure water as a blank for each measurement). At OD_{260}, an optical density of 1 corresponds to approximately 40 µg/ml RNA in an undiluted sample. Therefore, with the above dilution, an OD of 1 would correspond to 10 mg/ml. Thus, multiplication of the measured OD_{260} by 10 provides the RNA concentration in µg/µl. Purity of the RNA preparation was assessed as a ratio of OD_{260}:OD_{280}, in which a value of 2 indicates pure RNA, and a reading between 1.7 and 2.1 is considered acceptable purity.

A volume of RNA sample containing 10 µg RNA (as calculated using the method above) was used to synthesise cDNA, according to the standard method indicated by Promega. To this, a mixture of 14 µl 5x reverse transcriptase buffer, 7 µl 0.1 M DTT, 4 µl 20 mM dNTP, 2 µl 40 U/µl RNAse inhibitor and 1 µl 50 pmol random oligonucleotide primers was added, and a total volume of 66 µl was prepared (using nuclease-free water). The mixture was heated to 65°C for ten minutes and 4 µl 200 U/µl reverse transcriptase added, and the sample incubated at 42°C for 90 minutes. cDNA samples were stored at –80°C until required for rt-PCR analysis.

End-point rt-PCR analysis

End-point rt-PCR was carried out using specific primers for β-actin, PPARγ, C/EBPα, adipsin, IL-6 and the leptin. To 5 μl cDNA was added 10 μl 10x Taq polymerase buffer, 2 μl 5 mM dNTP, Mg^{2+} and 50 pmol each of forward and reverse primers (Figure 2.1), made up to 100 μl in H_2O. Samples were denatured at 95°C for 5 minutes, 0.5 μl Taq polymerase and 20 μl paraffin oil added, and cDNA amplified using specific primer conditions (Figure 2.1). Cycle lengths were: dissociation, 95°C, 30 seconds; annealing, primer specific (Figure 2.1), 1 minute; elongation, 72°C, 1 minute.

rt-PCR products were visualised on a 2% agarose gel. 2 g agarose was dissolved in 100 ml TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA), 4 μl ethidium bromide was added, and the gel was enabled to set [289]. Samples were loaded as 25 μl rt-PCR product mixed with 5 μl DNA loading buffer, and run at 80 V for 30 minutes. Agarose gels were visualised using a UVP ultraviolet lamp, box and software (Cambridge, UK).

Real-time rt-PCR analysis

Specific primer sequences for Taq-man® rt-PCR analysis were (i) purchased directly from the supplier (Applied Biosystems), (ii) designed using the software package Primer Express (Applied Biosystems), fulfilling all the criteria of primer design for this application or (iii) sequences from previously published papers [291-293]. Details of the primers used are shown in Figure 2.2. Real-time rt-PCR primers differ from those used for end-point rt-PCR because of the conditions used to carry out the reactions; whereas end-point rt-PCRs can be carried out at a range of temperatures (generally 56–64°C), for a varied number of cycles (determined by running the primers with a positive control through a number of cycles to find the linear growth period) and with different MgCl concentrations (generally < 5 mM), real-time rt-PCR conditions are fixed and require more stringent primer selection (which includes limits to

Primer	Sequence (5' – 3')	[MgCl] mM	Tm °C	No. of cycles
Actin F	AAGTACTCCGTGTGGATCGG	5	58	30
Actin R	CACCTTCACCGTTCCAGTTT			
Adipsin F	TGCACAGCTCCGTGTACTTC	0.5	56	30
Adipsin R	CACCTGCACAGAGTCGTCAT			
C/EBPα	GGGACTTAGGTGTTGGGGAT	0.5	56	35
C/EBPα	GGAAACCTGGCCTGTTGTAA			
IL-6 F	CCGGAGAGGAGACTTCACAG	2	58	30
IL-6 R	CAGAATTGCCATTGCACAAC			
ob F	CTCATGCCAGCACTCAAAAA	1	58	30
ob R	AGCACCACAAAACCTGATCC			
PPARγ F	TTTTCAAGGGTGCCAGTTTC	3	58	30
PPARγ R	TTATTCATCAGGGAGGCCAG			

Figure 2.1 – Primer sequences and running conditions for end point rt-PCR amplification of adipocyte gene products.

Forward (F) and reverse (R) primers for end point rt-PCR designed from published mRNA sequences and internet-based primer-design software. Shown are the sequences, magnesium chloride concentration, melting temperature (Tm) and number of cycles.

Primer	Sequence (5'–3')
Adipsin F	GCTATCCCAGAATGCCTCGTT
Adipsin R	GTTCCACTTCTTTGTCCTCGTATTG
β2-AR F	TGCCTATCCAGATGCACTGGTA
β2-AR R	CACAGCAAGTCTCCTCGGTGTA
β3-AR F	CCTCCTCCGTCTCCTTCTACCT
β3-AR R	CGCTTAGCCACAACGAACACT
C/EBPα F	TGCGCAAGAGCCGAGATAA
C/EBPα	CGGTCATTGTCACTGGTCAACT
GAPDH F	TCAACTACATGGTCTACATGTTCCAGTA
GAPDH R	CGCTCCTGGAAGATGGTGAT
IL-6F	CAGAATTGCCATCGTACAACTCTTTTCTCA
IL-6R	AAGTGCATCATCGTTGTTCATACA
ob F	ACCGCTCACCCACTTTTCAA
ob R	CGACCACTATCAGCAGCTACACA
PPARγ F	GGGTGAAACTCTGGGAGATTCTC
PPARγ R	TGCTCATAGGCAGTGCATCAG

Figure 2.2 – Primer sequences for real time rt-PCR.

Forward (F) and reverse (R) primers designed using Primer Express software for Taq-man rt-PCR, or from previously published papers.

the GC content, their positions within the primers and the number of contiguous Gs). Therefore, although the latter primers might work for end-point PCRs, the reverse is unlikely to be true. The Primer Express software often provides more than one possible set of primer pairs. As long as the sequence is unique, the set of primer pairs chosen should not affect the reaction.

Primers were prepared individually to a concentration of 80 nmol/ml, and forward and reverse primers were mixed 1:1 to give a final concentration of 40 nmol/ml of each primer. Reconstituted primers were stored at –20°C. Samples were run (at least) in duplicate, as 25 μl total volume per well in 96-well rt-PCR microtitre plates. This final volume consisted of 12.5 μl SYBR-green solution, 1.25 μl specific primer, 1 μl cDNA and 10.25 μl nuclease-free water. For each sample, a control mRNA, GAPDH (glyceraldehyde-phosphate dehydrogenase) [294], was run concurrently in duplicate. A negative control, nuclease-free water, for each set of primer pairs used was also included in each rt-PCR reaction. For each reported result, a minimum of three separate samples (i.e. the tissue or cells from three individual wells) were tested in duplicate. Amplification conditions using this method remain constant independent of the primer sequences used, providing the initial criteria are met. Reactions were run using the standard procedure specified by the ABI Prism 7000 software.

Data were analysed using a method described by Livak and Schmittgen for the relative quantification of real-time rt-PCR data [295]. This method, known as $2^{-\Delta\Delta Ct}$, calculates the relative changes in gene expression between two or more samples, in which one sample is nominated as a "baseline" (or calibrator) and all remaining samples are compared with this. Mathematical data and methods are shown in the appendix.

Northern analysis

RNA was isolated and quantified as described above. A 1% agarose gel was prepared by boiling 1 g agarose in 95 ml MOPS buffer (20 mM

MOPS, 5 mM sodium acetate and 1 mM EDTA), allowing the mixture to cool to approximately 60°C, adding 5 ml formaldehyde, and pouring. Once set, the gel was transferred to an electrophoresis tank in TBE buffer. RNA samples (20 μg total) were mixed with RNA loading buffer [50% formamide, 17% formaldehyde (36%), 6% glycerol in MOPS buffer, containing 1% ethidium bromide and bromophenol blue] to a total of 30 μl, denatured at 65°C for 10 minutes and loaded onto the gel. The gel was run at 80 V for 45 minutes, visualised under ultraviolet (UV) light, and washed for 30 minutes in DEPC□H_2O to remove excess formaldehyde. Concurrently, a gel-sized piece of nylon transfer membrane was soaked for 30 minutes in 2x SSC buffer (3 M sodium chloride and 0.3 M sodium citrate). The gel was removed from the DEPC□H_2O and placed upon five sheets of Whatmann filter paper, which had been pre-soaked in 10x SSC buffer, on a platform above a reservoir of 10x SSC buffer, with a paper towel wick connecting the gel to the buffer. The nylon membrane was placed upon the gel, ensuring that no air bubbles were present. On top of the membrane five sheets of Whatmann filter paper, also pre-soaked in 10x SSC, and two dry blotting pads were placed. The gel/membrane was left to contact blot during an overnight incubation at room temperature [289].

The gel and membrane were removed from the blotting structure. Transfer of RNA was assessed by UV photography, and the membrane cross-linked twice in an UV crosslinker. The membrane was then baked dry at 80°C for 1 h and stored for northern analysis.

Plasmids for β2- and β3-adrenoceptors were cultured overnight in an *Escherichia coli* host in LB broth (1% tryptone, 0.5% yeast extract and 1% NaCl, in water), at 37°C and isolated by alkaline lysis. The culture was centrifuged at 4000 rpm for 10 minutes, and the pellet reconstituted in an ice-cold solution of 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA. 1% SDS in 0.2 N NaOH was added, and the sample was mixed and stored on ice. An ice-cold solution of 5 M potassium acetate and glacial acetic acid was added, and the mixture incubated on ice for five minutes. This was centrifuged at 10000 rpm for five minutes at 4°C, and

the supernatant solution added to an equal volume of phenol:chloroform:isoamylalcohol 25:25:1. This was centrifuged for two minutes at 10000 rpm at 4°C and the double-stranded DNA from the upper phase was precipitated with two volumes of absolute ethanol. The sample was centrifuged at 10000 rpm for ten minutes, the pellet was air-dried for ten minutes and then dissolved in 50 µl TBE buffer. The nucleic acid content was assessed by OD_{260} measurement. The DNA probes were isolated from the plasmids using restriction enzymes. The plasmid containing the β2-adrenoceptor insert was incubated overnight with *EcoR1* and *Pst1*, and the β3-adrenoceptor plasmid with *EcoR1* and *Xba1*. The products were separated on a 1% agarose gel (1 g agarose in 100 ml TBE buffer). Samples were loaded as 25 µl sample and 5 µl DNA loading buffer (30% glycerol and 70% water, containing bromophenol blue and 1% ethidium bromide), and the gel was run at 80 V for 1 h [289]. Following visualisation, the insert bands were excised and the agarose that had been removed was weighed. The agarose was digested using a Qiagen plasmid kit (Qiagen, Crawley, UK) and the insert liberated. The final pellet was dissolved in 10 mM Tris·Cl pH 8.5. Nucleic acid content was assessed by measurement at OD_{260}.

The RNA membrane was placed in a hybridisation tube with 10 ml of hybridisation buffer (consisting of 50 mM PIPES pH 6.5, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS) and 120 µl sheared salmon sperm DNA (Ambion). The membrane was pre-hybridised for 1 h at 65°C. The hybridisation probe mixture was prepared as 1 µl plasmid insert in 9 µl H_2O, and boiled for five minutes. 1 µl each of dATP, dGTP and dTTP and 2 µl hexanucleotides in 10x reaction buffer was added on ice. To this, 5 µl ^{32}P-dCTP and 1 µl klenow enzyme was added, and the mixture incubated for 1 h at 37°C. The probe was then purified using a spin column, washed with STE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA and 150 mM NaCl), and spun at 2000 rpm for two minutes. The purified probe was boiled for ten minutes, and added to the membrane and hybridisation buffer. The probe and membrane were incubated overnight

at 65°C, rotating. Following incubation, the membrane was washed for ten minutes in wash buffer (5% SDS in 0.5x SSC) at room temperature, and twice at 65°C for 15 minutes. The membrane was wrapped in Clingfilm, and taped inside an x-ray film cassette adjacent to unexposed x-ray film. The cassette was stored at –80°C for two weeks, before the film was developed. The membrane was stripped of probe by incubating in boiling wash buffer for ten minutes, and ensuring that all radioactive label was removed. The hybridisation process was then repeated for the second probe.

Protein isolation

Protein for western blotting was isolated from 3T3-L1 adipocytes using two methods. For proteins of the insulin signalling machinery (insulin receptor subunit-β (IRβ), IRS-1 or the phosphorylation of IRS-1) the adipocytes were digested and homogenised using RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM pefabloc, 1 mM activated NA_3VO_4, 1 mM sodium fluoride, 1 μg/ml aptotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). Homogenates were centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatant solution retained [296, 297].

Subcellular isolation of the glucose transporters GLUT1 and GLUT4 was carried out by digesting 3T3-L1 adipocytes with 5 ml per flask HES buffer (20 mM HEPES pH 7.4 containing 1 mM EDTA, 255 mM sucrose and a cocktail of pre-prepared protease inhibitors). Homogenised cells were centrifuged at 19 000 g for 20 minutes at 4°C (Figure 2.3). The supernatant solution (total internal membranes) was removed and centrifuged at 150 000 g for 60 minutes at 4°C and the pellets were gently reconstituted in 100 μl HES buffer. The pellets from the original 19 000 g spin were resuspended in 2 ml HES buffer, and layered onto 1 ml 1.12 M sucrose in HES buffer. This was centrifuged at 100 000 g for 1 h at 4°C. The interface was collected and resuspended in HES buffer, and

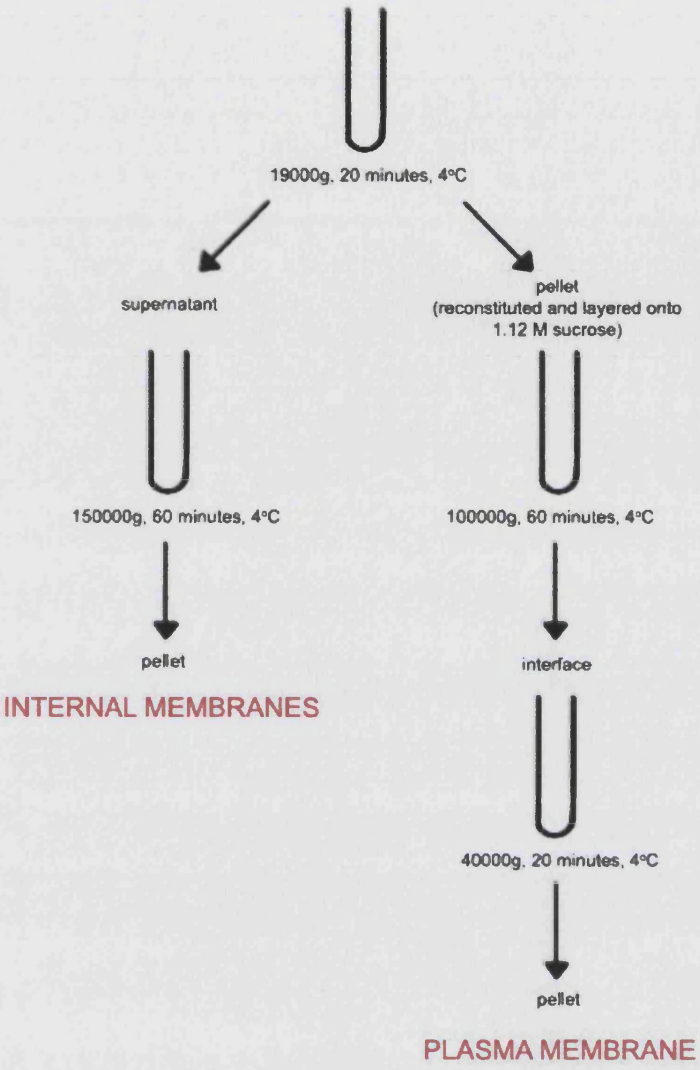

Figure 2.3 – Centrifugal separation of the internal and plasma membranes of 3T3-L1 adipocytes

Homogenised 3T3-L1 adipocytes (in HES buffer) were centrifuged appropriately to separate both the internal and plasma membranes. Where required, pellets and interfaces were reconstituted in HES buffer, at volumes described within the text.

centrifuged at 41 000 g for 20 minutes at 4°C. The pellets were reconstituted in 50 μl HES buffer [298].

Protein concentration was assessed using the Lowry method [299]. 5 μl of sample or standard (BSA in HES buffer at concentrations of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml and 0.0313 mg/ml) was added to a microtitre plate, incubated with Bio-Rad protein assay reagent A for 15 minutes and 200 μl reagent B for a further 15 minutes. Absorbance was measured at 650 nm.

Western analysis

Protein detection was performed using ECL (enhanced chemiluminescence), according to the manufacture's instructions. Briefly, the system uses the emission of light from luminol in the presence of HRP (horseradish peroxidase) and hydrogen peroxide, as well as chemical enhancers (such as phenol). Exposure of autoradiography paper to the reaction enables the visualisation of light emission (following development of the paper). To achieve this, protein is bound to a membrane and incubated with a primary antibody. A secondary HRP-conjugated antibody binds to the primary antibody, and this is exposed to detection reagents containing luminol and hydrogen peroxide.

Protein samples extracted using either of the methods described above were diluted to a concentration of 20 μg/μl in RIPA or HES buffer (as appropriate). A sodium dodecyl sulphate polyacrylamide (SDS-PAGE) separating gel was prepared (10% polyacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED), with a stacking gel (5% polyacrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED). A 20 μl volume of sample was mixed with 5 μl of sample loading buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.25% bromophenol blue and 5% β-mercaptoethanol) and either boiled for three minutes (samples in RIPA buffer) or incubated at 37°C for ten minutes (samples in HES buffer) prior to loading onto the gel. Gels were run at 100 V until sufficient migration

had occurred, in a running buffer of 25 mM Tris-base, 0.192 M glycine and 0.1% SDS [289].

The gel was removed, briefly washed in distilled water and soaked in transfer buffer (48 mM Tris-base, 39 mM glycine and 0.037% SDS) for 20 minutes. A gel-sized piece of ECL membrane was also incubated for 20 minutes in transfer buffer. Protein was transferred from the gel to the membrane using a semi-dry electrophoretic method (Multiphor II, Pharmacia, Uppsala, Sweden). The membrane was placed upon the electrode on top of six sheets of filter paper that had been soaked in transfer buffer, the gel was placed on top of the membrane and covered with a further six sheets of soaked filter paper. The negative electrode was placed upon the construct and the transfer run at 20 V for 30 minutes [289].

The membrane was blocked for 1 h at room temperature in a mixture of phosphate buffered saline/Tween 20 (PBST, PBS containing 0.5% Tween) with 5% Marvel powdered milk, and washed twice in PBST (ten minutes per wash). The primary antibody, diluted 1:1000 in PBST, was incubated with the membrane overnight at room temperature. The membrane was washed three times in PBST (ten minutes per wash) and incubated for 2 h with a secondary antibody (HRP-conjugated, 1:5000 in PBST) at room temperature. The membrane was washed three times as before and incubated with ECL+plus reagent for two minutes. Excess reagent was removed by enabling it to run off the membrane, which was wrapped in Clingfilm. The membrane was exposed to x-ray film for 15 seconds to two minutes (depending on the antibody used) and the film developed.

Assays

Assays for IL-6 and leptin were carried out in accordance with the manufacturer's instructions. Both assays are two-site ELISAs, with polyclonal antibodies pre-bound to the microtitre plate. Standards and samples are incubated and a second, HRP-conjugated antibody is added. A colour chromogen solution (tetramethylbenzidine) is added, and colour

change (equivalent to target concentration) is measured at 450 nm, with wavelength correction at 540 nm. The published intra- and inter- assay variability for these assays are 4.7% and 6.8% (IL-6) and 3.8% and 5.8% (leptin), with sensitivities of < 3.1 pg/ml (IL-6) and < 20 pg/ml (leptin).

Glycerol accumulation was used as a measure of lipolysis, using the reagent GPO Trinder according to the manufacturer's instructions. 40 µl sample or standard (625, 312.5, 156.3, 78.1, 39.1, 19.5 and 9.8 µg/ml Sigma glycerol standard) was added to 200 µl GPO Trinder, incubated for five minutes and absorbance measured at 570 nm.

Chapter 3 – Adipose Tissue Release of IL-6

Introduction

Adipocytes are unusual among endocrine tissues in that they are not specialised for the release of one major hormone, as, for example, pancreatic β-cells are for insulin or adrenal glands are for adrenalin, but they release numerous adipokines. Furthermore, the release of adipokines is also dependent upon the location of the adipose tissue depot, as well as the fat content of the organism. Finally, adipokines do not appear to have a single mechanism of release, but each investigated thus far has a unique pattern of control and secretion.

Leptin, for example, is expressed in significantly greater amounts in subcutaneous depots compared with visceral [300, 301]. A variety of stimuli have a differential effect on the expression and secretion of leptin from these depots: dexamethasone increases release from visceral tissue only, despite increasing the level of mRNA in both depots; insulin increases subcutaneous release without affecting expression in either depot [119]; and transforming growth factor-β1 (TGF-β1) inhibits leptin release more effectively from visceral depots than it does from subcutaneous [120]. In spite of this difference in release and expression, leptin secretion does appear to be closely linked with mRNA levels, and to the lipid volume of the adipocyte [34].

Investigation into the secretory pathway of leptin revealed the many different pathways that are used in the adipocyte. Prior to release, leptin is stored in intracellular pools within the adipocyte, as are numerous other proteins, but these pools are separate from those containing GLUT4, LPL or angiotensinogen [287, 288, 302]. This indicates a number of different mechanisms through which adipocytes can transport products to the cell surface, even after the same stimulus (insulin is an effective stimulant for the release of each of these examples) [303]. Furthermore, adiponectin release (also stimulated by insulin) follows a different pathway to that seen for GLUT4 [187]. Leptin secretion from adipocytes is a steady-state process (under normal

conditions) [288], and does not require *de novo* synthesis of the protein. This implies that, although leptin is released at a constant rate, significant stores are maintained within the adipocyte to enable a response to stimulation [166].

Clearly, therefore, adipocytes use a variety of secretory pathways to release adipokines. Although the different secretory pathways for leptin and adiponectin have been identified, the molecular control of release for other adipokines, including IL-6, has not been investigated. In spite of a range of knowledge concerning factors that affect its release, including β-adrenoceptor agonists or IL-1β (both of which have been reported to stimulate IL-6 release from adipocytes) [235, 256], little is known about the mechanism by which this release occurs. Increased IL-6 release is observed from the visceral tissue of obese subjects, but whether this is true of normal weight subjects is unknown [236].

Studies in humans have shown that obese subjects have higher circulating levels of IL-6 [243] and that in the obese, visceral adipose tissue releases greater levels of IL-6 than does subcutaneous [236]. If a murine model is to recapitulate human obesity, at least for the purposes of autocrine/paracrine IL-6 release, a similar pattern of IL-6 release must be confirmed. Therefore, the studies presented here were designed to confirm whether this was true. Furthermore, an investigation into IL-6 release from lean-animal-derived adipose tissue was carried out to determine whether release was affected by the obese state of the animal, which might be expected considering that circulating IL-6 is higher in the obese. Finally, the mechanism of release of IL-6 from adipose tissue was examined, to test whether IL-6 is stored within adipocytes prior to release, as are a number of other adipokines.

Thus, the aims of this chapter are to investigate:
(i) The effect of obesity on IL-6 levels in a rodent model of obesity.
(ii) The depot specific difference in IL-6 release from lean and obese animals.

(iii) The mode of secretion of IL-6, both constitutive and regulated, using the Golgi-disrupting molecule brefeldin A to abolish intracellular transport and, therefore, secretion, as well as an inhibitor of transcription, cycloheximide, to prevent protein production.

Methods

As described in Chapter 2, 12-week-old *ob*$^{-/-}$ mice, or their wild-type cousins (C57BL6/J), were weighed, tail bled and killed. Unfortunately, facilities were not available for the accurate measurement of body-fat distribution, such as magnetic resonance imaging (MRI) or DEXA. Both these techniques enable an accurate analysis of whole-body tissue distribution; the adipose tissue weight measurements presented here are crude analyses of easily removed tissue, to give an indication of relative adiposity. Adipose tissue was removed from two major sources: subcutaneous tissue was extracted from beneath the skin, but without entering the body cavity, and epididymal adipose tissue (or omental or visceral: these three terms are used in this thesis and refer, in mice, to the same tissue depot) was removed from the body cavity and surrounding all the visceral organs. Obvious blood vessels within the adipose tissue were avoided. Tissue from up to six weight-matched animals was pooled (to minimise inter-animal variation) and washed in warm PBS to remove any non-adipose tissue, and fragments of tissue weighing between 50 mg and 130 mg were separated and placed in 12-well plates. Tissue fragments were incubated in 2 ml per well Cell-gro, supplemented with the cytokine IL-1β, the β3-adrenoceptor agonist CL316243, the Golgi-disrupting brefeldin A or the transcription inhibitor cycloheximide, or with a combination of IL-1β or CL316243 with either brefeldin A or cycloheximide. A subset of tissue was also incubated in Cell-gro alone to act as a basal control. Tissue was incubated for 5 h or 24 h, after which the media was removed for assay, and tissue stored for RNA isolation, as described in Chapter 2. In addition to adipose tissue, fragments of liver were removed and incubated for 5 h in Cell-gro to measure the basal release of IL-6 from another tissue source.

A similar set of experiments was also carried out on mature 3T3-L1 adipocytes, differentiated using dexamethasone, IBMX, insulin and BRL 49653, as described in Chapter 2. Cells were differentiated in 6-

well plates, and treated for 24 h with 1 ml of Cell-gro containing the same treatments as the tissue fragments.

The blood and supernatant solutions were assayed using a commercially available murine IL-6 ELISA (R & D Systems). The IL-6 concentrations from the tissue supernatant assay were divided by the weight of the original tissue fragment, to give the total IL-6 release per gram of tissue. Adipose tissue was homogenised and RNA isolated for real-time rt-PCR analysis as described in Chapter 2. All data are shown as median and interquartile range, and were obtained from a minimum of six individual tissue fragments per treatment, and a minimum of two separate experiments. The *n* values for the IL-6 release graphs from explanted adipose tissue refer to the number of individual wells from which samples were taken. Significance was calculated using the Mann–Whitney U test, with two-tailed *p* values below 0.05 considered significant.

Results

Murine adiposity and circulating IL-6

As with humans, an increase in body mass in mice was accompanied by an increase in circulating IL-6 levels (Figure 3.1). Leptin-deficient $ob^{-/-}$ animals were significantly heavier than wild-type counterparts of the same age [55.6 (48.9–60.9) vs. 27.1 (24.8–37.1) g, $p< 0.001$], and had significantly higher plasma IL-6 levels [8.6 (4.6–24.5) vs. 2.8 (2.5–5.9) pg/ml, $p< 0.001$]. The total mass of adipose tissue obtained from each set of animals indicated that a considerable proportion of the weight increase observed in the $ob^{-/-}$ animals was due to an increase in adipose tissue, as opposed to other sources. A crude estimation of subcutaneous and visceral adipose tissue from lean animals comprised a mean of 0.8% and 1.7% of total mass, respectively, whereas in obese animals these figures were 6.1% and 6.8%, respectively. Because this measurement was obtained only through a measurement of the mass of removed tissue, it cannot be compared with more accurate calculations of adipose tissue distribution.

Depot- and obesity-specific differences in IL-6 secretion

Having established that obese mice had a higher concentration of circulating IL-6 than did lean animals, the release of IL-6 from adipose tissue depots in both lean and obese mice was measured. As shown in Figure 3.2, basal (unstimulated) IL-6 release differed depending on both the location of the depot and the total body mass of the animal. Adipose tissue release of IL-6 from lean animals during a 24 h incubation showed no significant difference between subcutaneous and visceral tissue [16.7 (13.3–30.5) vs. 27.3 (25.0–39.3) ng/ml per gram of tissue, $p= 0.37$], whereas visceral tissue from obese animals released significantly more IL-6 over 24 h than did subcutaneous [58.1 (48.8–66.3) vs. 20.9 (11.5–24.2) ng/ml per gram tissue, $p= 0.004$].

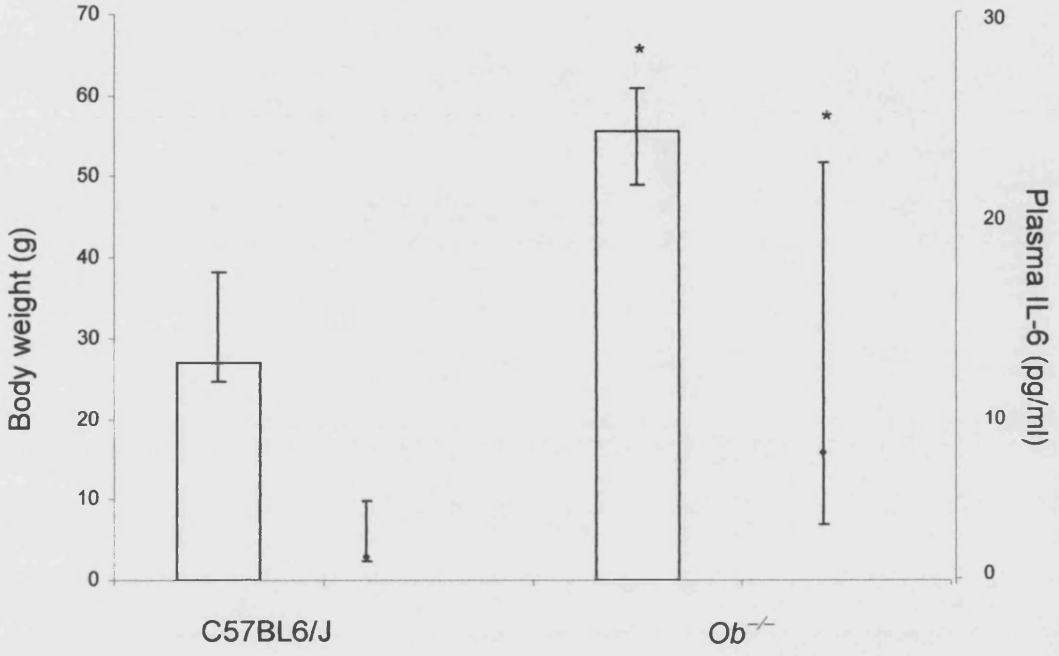

Figure 3.1 – Weight and circulating IL-6 levels of C57BL6/J and *ob*$^{-/-}$ mice.

Total body weight (columns, left hand axis) and circulating IL-6 (points, right hand axis) of 12-week-old C57BL6/J and *ob*$^{-/-}$ mice. Data presented as median and interquartile range.

* $p < 0.001$

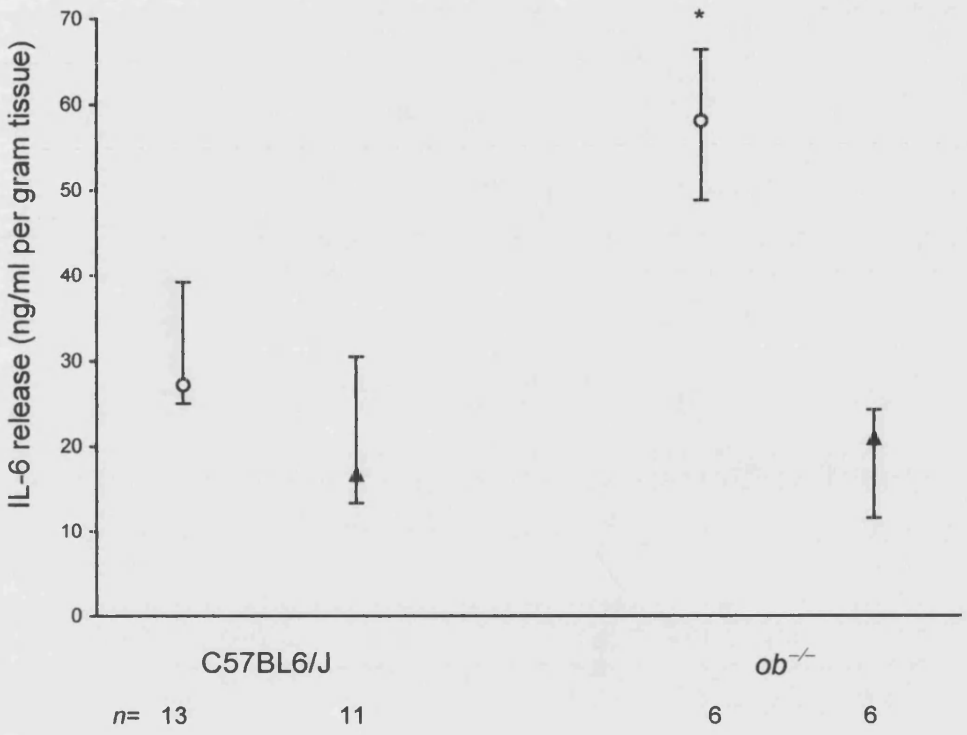

Figure 3.2 – 24 h accumulation of IL-6 release from unstimulated C57BL6/J and *ob*$^{-/-}$ adipose tissue explants.

Basal (unstimulated) release of IL-6 from C57BL6/J and *ob*$^{-/-}$ adipose tissue explants from visceral (O) or subcutaneous (▲) depots. Data presented as median and interquartile range of IL-6 released into supernatant over 24 h, corrected to 1 g of tissue.

* p= 0.004 vs. *ob*$^{-/-}$ subcutaneous, and p= 0.015 vs. C57BL6/J visceral.

When comparing the depot-specific release from lean and obese animals, clear location-dependent differences were observed. IL-6 release from subcutaneous tissue did not vary significantly, immaterial of whether the animal was obese or not (16.7 vs. 20.9 ng/ml per gram tissue lean vs. obese, p= 1). Visceral release from obese animals, however, was more than twice that of lean animals (58.1 vs. 27.3 ng/ml per gram, p= 0.015).

The incubation of tissue from C57BL6/J animals for 5 h follows a similar pattern to that of the 24 h incubation (Figure 3.3), with no significant difference between IL-6 release from subcutaneous or visceral depots (6.4 ng/ml per gram tissue subcutaneous vs. 6.3 ng/ml per gram tissue visceral, p= 0.903). Notably, the rate of IL-6 release differs between the tissues during these incubation periods. Assuming a steady-state release of IL-6, subcutaneous tissue from C57BL6/J animals is 1.28 ng/h per gram of tissue over 5 h, compared with 0.69 ng/h per gram of tissue over 24 h and, from obese animals, 0.87 pg/h per gram of tissue. Visceral tissue from C57BL6/J mice released 1.26 pg/h IL-6 per gram of tissue over 5 h and 1.13 pg/h per gram of tissue over 24 h, whereas obese mice released 2.42 pg/h per gram of tissue over 24 h.

As a comparison with other cellular sources of IL-6, fragments of liver (from C57BL6/J) were also incubated for 5 h in Cell-gro to assess relative levels of release. Liver (0.346 ng/ml per gram tissue) released significantly less IL-6, in the absence of stimulation, than either subcutaneous (6.4 ng/ml per gram tissue, p= 0.034) or visceral (6.3 ng/ml per gram tissue, p= 0.009) adipose tissue.

Stimulated IL-6 release

The incubation of adipose tissue for 24 h with the immunological stimulant IL-1β or the β3-adrenoceptor agonist CL316243 affected IL-6 release, depending on both the adipose tissue depot and the obese state of the host animal.

In lean animals (Figure 3.4), the only observed effect was from visceral tissue in response to IL-1β [27.3 ng/ml per gram tissue basal, vs.

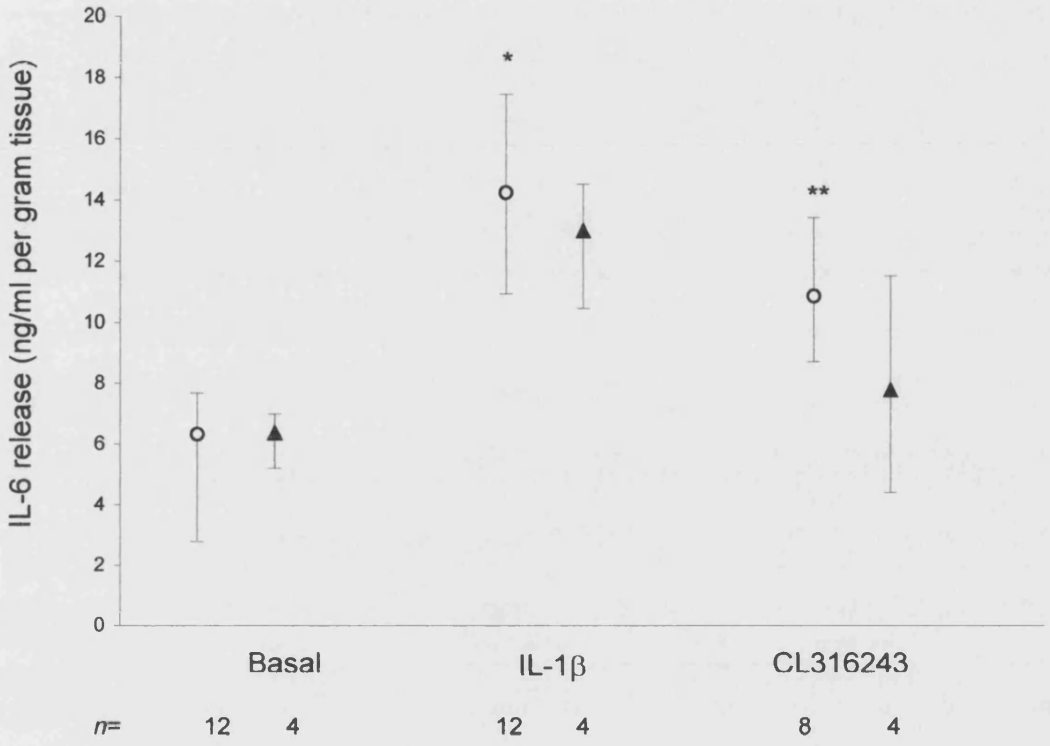

Figure 3.3 – 5 h accumulation of IL-6 release from C57BL6/J adipose tissue explants incubated with IL-1β or CL316243.

The release of IL-6 from C57BL6/J adipose tissue explants from visceral (O) or subcutaneous (▲) depots exposed to 5 h incubation with IL-1β, CL316243 or no stimulation (basal). Data presented as median and interquartile range of IL-6 released into supernatant over 2 h, corrected to 1 g of tissue.

* p= 0.001; ** p= 0.017; both vs. basal (same depot).

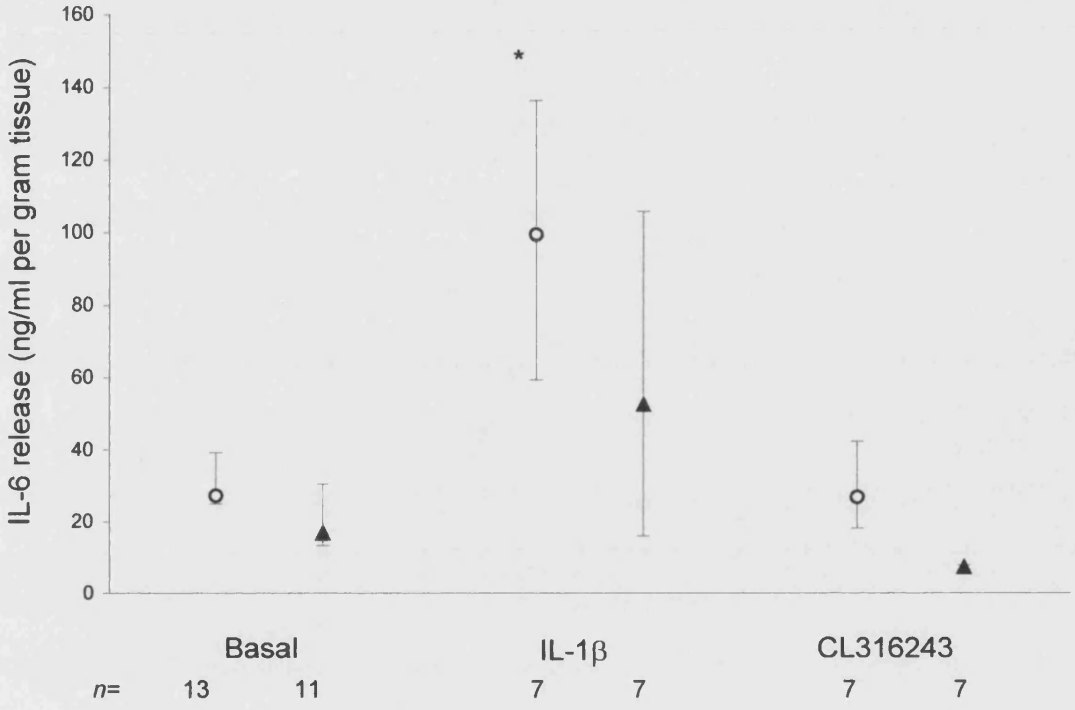

Figure 3.4 – 24 h accumulation of IL-6 release from C57BL6/J adipose tissue explants incubated with IL-1β or CL316243.

The release of IL-6 from C57BL6/J adipose tissue explants from visceral (O) or subcutaneous (▲) depots exposed to 24 h incubation with IL-1β, CL316243 or no stimulation (basal). Data presented as median and interquartile range of IL-6 released into supernatant over 2 h, corrected to 1 g of tissue.

*p= 0.009 vs. basal.

99.5 (59.4–136.4) ng/ml per gram tissue IL-1β stimulated, p= 0.009]. There was no significant effect of IL-1β on subcutaneous tissue [52.5 (15.9–105.8) ng/ml per gram tissue stimulated, vs. 16.7 ng/ml per gram tissue basal, p= 0.347]. Using CL316243 as a β-adrenoceptor stimulant affected neither subcutaneous [7.3 (6.7–7.9) ng/ml per gram tissue, p= 0.121] nor visceral [26.8 (18.1–42.3) ng/ml per gram tissue, p= 0.85] tissue release of IL-6.

Tissue from the $ob^{-/-}$ animals was more responsive than that from lean animals (Figure 3.5). Incubation with IL-1β caused an increase in IL-6 release from both visceral [58.1 ng/ml per gram tissue basal vs. 91.3 (79.7–103.7) ng/ml per gram tissue IL-1β stimulated, p= 0.004] and subcutaneous [20.9 ng/ml per gram tissue basal vs. 56.5 (44.1–87.1) ng/ml per gram tissue IL-1β stimulated, p= 0.006] depots over 24 h. In contrast to the lack of effect on tissue from lean mice, CL316243 significantly increased IL-6 release from visceral tissue from obese animals [89.6 (80.7–90.4) ng/ml per gram tissue, p= 0.01]. It had no effect on subcutaneous tissue from the same animals [22.3 (16.8–24.2) ng/ml per gram tissue, p= 0.873].

A similar pattern was observed in the response of adipose tissue from C57BL6/J adipose tissue exposed to the agents for 5 h (Figure 3.3). IL-6 release from visceral depots was stimulated by IL-1β (basal 6.3 vs. IL-1β 14.2 ng/ml per gram tissue, p= 0.001) but subcutaneous depots were unaffected (basal 6.4 vs. IL-1β 13.0 ng/ml per gram tissue, p= 0.149). 5 h incubation with CL316243 also stimulated visceral IL-6 release (10.9 ng/ml per gram tissue, p= 0.017) but not subcutaneous (8.1 ng/ml per gram tissue, p= 0.564).

Inhibition of IL-6 release

The incubation of adipose tissue from either depot or animal source with the Golgi-disrupting brefeldin A or transcription-inhibitor cycloheximide completely abrogated IL-6 release (Figures 3.6 and 3.7). C57BL6/J release was reduced to 1.8 (1.7–2.2) ng/ml per gram tissue (p= 0.009) for

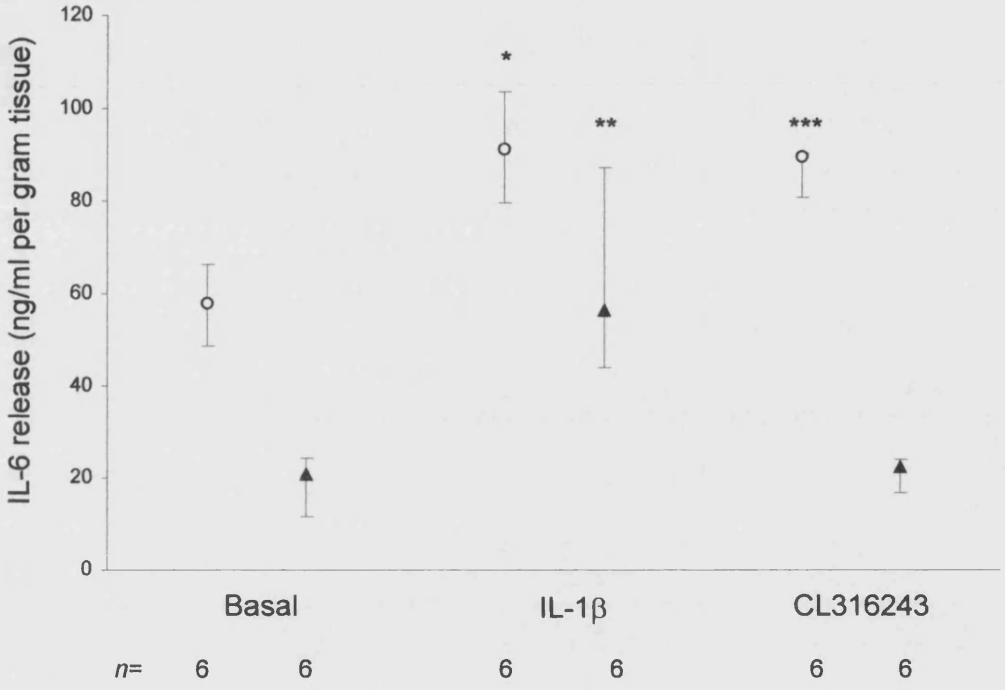

Figure 3.5 – 24 h accumulation of IL-6 release from *ob*$^{-/-}$ adipose tissue explants incubated with IL-1β or CL316243.

The release of IL-6 from *ob*$^{-/-}$ adipose tissue explants from visceral (O) or subcutaneous (▲) depots exposed to 24 h incubation with IL-1β, CL316243 or no stimulation (basal). Data presented as median and interquartile range of IL-6 released into supernatant over 24 h, corrected to 1 g of tissue.

* p= 0.004; ** p= 0.006; *** p= 0.01; all vs. basal (same depot).

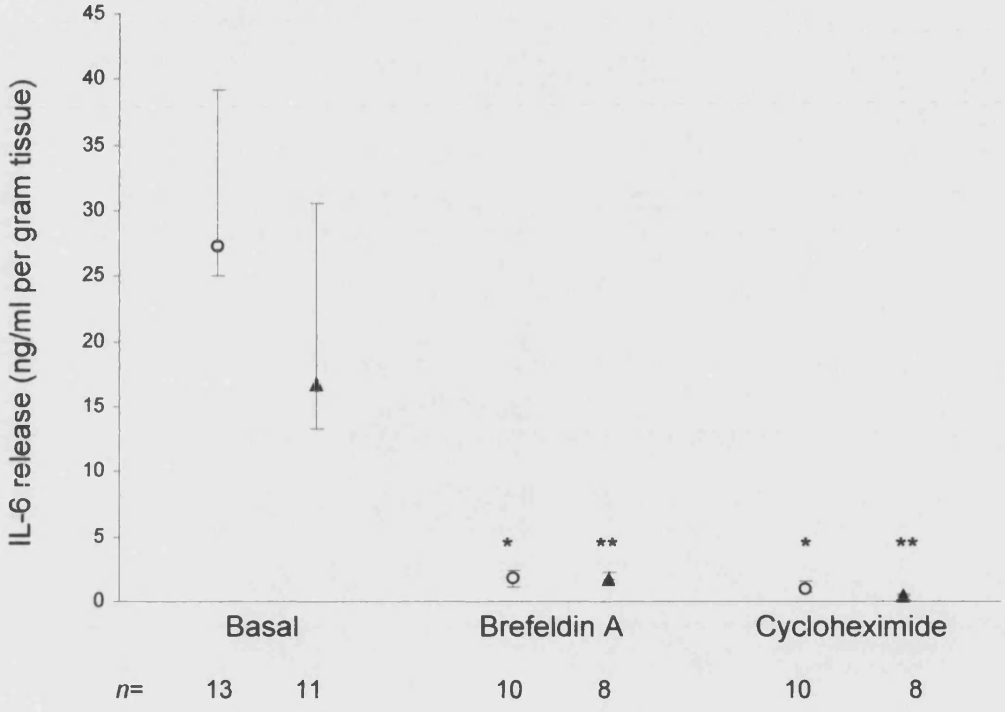

Figure 3.6 – 24 h accumulation of IL-6 release from C57BL6/J adipose tissue explants incubated with brefeldin A or cycloheximide.

The release of IL-6 from C57BL6/J adipose tissue explants from visceral (O) or subcutaneous (▲) depots exposed to 24 hr incubation with brefeldin A, cycloheximide or no stimulation (basal). Data presented as median and interquartile range of IL-6 released into supernatant over 24 h, corrected to 1 g of tissue.

* p= 0.002; ** p= 0.009; both vs. basal (same depot).

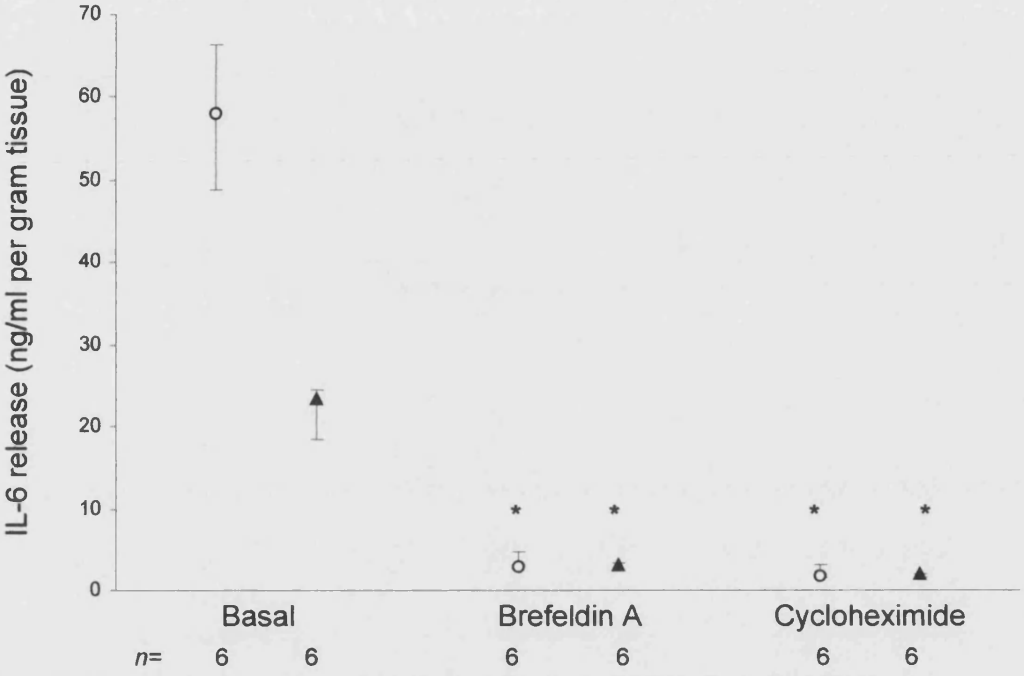

Figure 3.7 – 24 h accumulation of IL-6 release from *ob*$^{-/-}$ adipose tissue explants incubated with brefeldin A or cycloheximide.

The release of IL-6 from *ob*$^{-/-}$ adipose tissue explants from visceral (O) or subcutaneous (▲) depots exposed to 24 h incubation with brefeldin A, cycloheximide or no stimulation (basal). Data presented as median and interquartile range of IL-6 released into supernatant over 24 h, corrected to 1 g of tissue.

*p= 0.02

subcutaneous and 1.9 (1.1–2.5) ng/ml per gram tissue (p= 0.002) for visceral by brefeldin A, and to 0.4 (0.4–0.5) ng/ml per gram tissue (p= 0.009) and 1.1 (0.7–1.6) ng/ml (p= 0.002) per gram tissue respectively by cycloheximide. The effect was the same in tissue from $ob^{-/-}$ animals, in which brefeldin A reduced IL-6 release to 3.9 (3.0–4.8) ng/ml per gram tissue (p= 0.02) from subcutaneous and 3.0 (2.5–3.4) ng/ml per gram tissue (p= 0.02) from visceral, and cycloheximide to 1.9 (1.6–3.1) ng/ml per gram tissue (p= 0.02) and 1.7 (1.6–2.1) ng/ml per gram tissue (p= 0.02).

The incubation of C57BL6/J explants with brefeldin A or cycloheximide for 5 h produced similar results. Subcutaneous tissue IL-6 release was reduced to 0.12 (0.09–1.14) ng/ml per gram (basal 6.4 ng/ml per gram, p= 0.021) by brefeldin A, and to 0.17 (0.15–0.19) ng/ml per gram (p= 0.021) by cycloheximide. Release by visceral tissue was reduced to 0.07 (0.06–0.08) ng/ml per gram (basal 6.3 ng/ml per gram, p< 0.001) by brefeldin A and to 0.20 (0.17–0.29) ng/ml per gram tissue (p< 0.001) by cycloheximide.

Subcutaneous tissue from obese animals that was incubated with IL-1β and brefeldin A released 3.8 (3.7–6.6) ng/ml IL-6 per gram tissue (p= 0.07); with IL-1β and cycloheximide, 2.0 (1.7–2.2) ng/ml per gram tissue (p= 0.02); with CL316243 and brefeldin A, 3.7 (2.8–4.0) ng/ml per gram tissue (p= 0.02); and with CL316243 and cycloheximide, 1.6 (1.5–1.6) ng/ml per gram tissue (p= 0.02). IL-6 release from visceral tissue from the same animals was also significantly inhibited; IL-1β and brefeldin A reduced release to 6.4 (6.3–6.6) ng/ml per gram; IL-1β and cycloheximide, to 1.7 (1.7–4.8) ng/ml per gram; CL316243 and brefeldin, to A 3.5 (3.0–5.5) ng/ml per gram; and CL316243 and cycloheximide, to 1.6 (1.5–2.2) ng/ml per gram (all p= 0.02).

Cell-line release of IL-6

In addition to measuring IL-6 release from adipose tissue explants, the murine cell line 3T3-L1 was used to measure IL-6 release. Fully

differentiated adipocytes were treated with Cell-gro supplemented with IL-1β, CL316243, brefeldin A or cycloheximide. Incubation with IL-1β significantly increased IL-6 release over 24 h [basal 1.3 (0.9–1.6) vs. IL-1β 1.8 (1.8—2.0) ng/ml, p= 0.02], whereas CL316243 had no effect [1.56 (1.1–1.7) ng/ml, p= 0.79]. Both brefeldin A [0.61 (0.54–0.64) ng/ml, p= 0.046] and cycloheximide [0.027 (0.024–0.042) ng/ml, p= 0.02] significantly inhibited 3T3-L1 IL-6 release.

IL-6 expression in adipose tissue

The expression of IL-6 in adipose tissue closely mirrored its release (Figure 3.8). There was no significant difference in visceral expression compared with subcutaneous in lean adipose tissue [visceral expression 1.6 (0.5–6.2) fold that of subcutaneous, p= 0.309]. Nor was there a difference between the expression of IL-6 from subcutaneous tissues of lean or obese animals [obese subcutaneous expression 1.7 (1.0–1.9) fold that of lean, p= 0.944]. As with the release data, however, IL-6 expression was significantly greater in visceral tissue from obese animals compared with mRNA levels from both lean [3.6 (2.7–4.0) fold that of C57BL6/J subcutaneous, p= 0.001] and obese subcutaneous depots (p= 0.001), but not compared with lean visceral (p= 0.139).

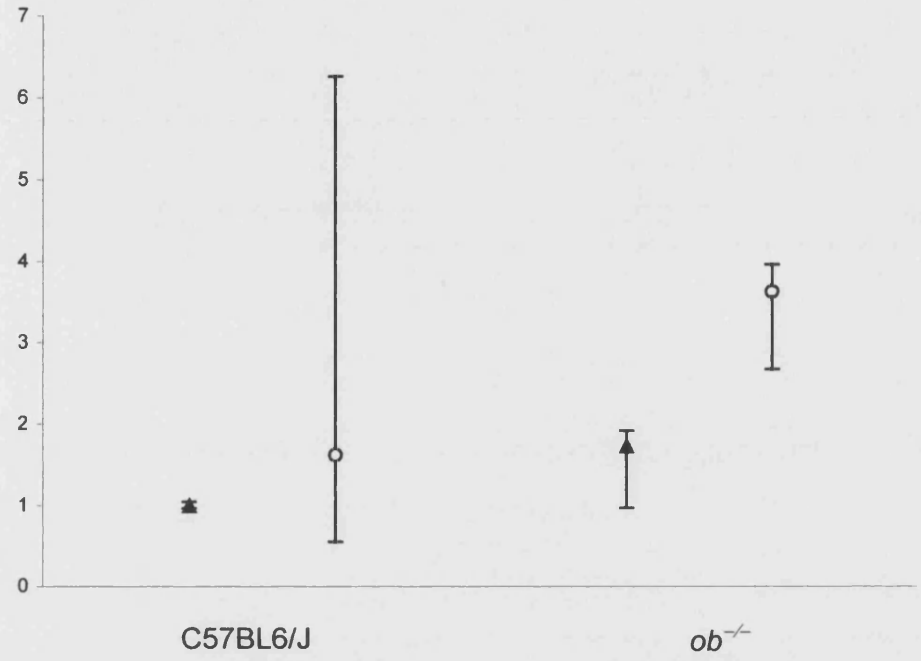

Figure 3.8 – C57BL6/J and *ob*$^{-/-}$ adipose tissue explant expression of IL-6.

The semi-quantitative expression of IL-6 from C57BL6/J and *ob*$^{-/-}$ subcutaneous (▲) and visceral (O) adipose tissue. Expression is corrected to GAPDH mRNA levels and displayed as median (interquartile range) fold-increase compared with C57BL6/J subcutaneous levels.

* p= 0.001 vs. *ob*$^{-/-}$ subcutaneous.

Discussion

Prior to making any comparisons to the situation in humans, it was important to establish that obesity in mice is characterised by the same rise in circulating IL-6 levels that is observed in human obesity [103, 304, 305]. This study demonstrates that mice do exhibit increased circulating IL-6 in the obese state. Furthermore, these data establish that adipose tissue is a source of significant IL-6 production, which is likely to contribute to increased circulating levels. Calculations in human subjects, which were based on an assumption that subcutaneous adipose tissue released similar amounts of IL-6 to other depots – which the data here (and elsewhere [236]) show not to be the case (in the obese) – indicated that up to 35% of circulating IL-6 was adipose tissue derived [103]. Whether similar arterio-venous sampling of adipose tissue is possible in mice has not been examined. Visceral adipose tissue from obese animals not only had an alteration in the production of IL-6 but also had altered sensitivity to the stimulation of IL-6 production. These data demonstrate that IL-6 production by adipose tissue is constitutive, requires transcription and is trafficked through the adipocyte in a Golgi-dependent manner, although, unlike other adipokines, it is not stored within the cell prior to release.

The studies described here have used the leptin-deficient $ob^{-/-}$ mouse as a model for murine obesity. These animals exhibit weight gain and hypertrophy from weaning compared with wild-type C57BL6/J [306]. In spite of being the product of a gene knockout, the $ob^{-/-}$ mouse is a good model of obesity to investigate the release of IL-6. A lack of leptin has a number of serious consequences for the organism, in addition to the increase in body weight and accompanying insulin resistance, such as infertility [307], reduced metabolic rate [308], impaired liver function [309] and increased anxiety [310]. However, leptin appears to be a true endocrine factor, although its autocrine/paracrine effects on adipose tissue are disputed, and might depend on the adipose tissue source [126, 311, 312]. The data presented here clearly indicate that IL-6 release is

unaffected by leptin deficiency, or demonstrate that any effect is insufficient to inhibit the rise in circulating IL-6 that is observed in obesity. Therefore, despite the inherent physiological deficiencies, the $ob^{-/-}$ model of obesity might be an acceptable alternative to diet-induced obesity for the purposes of measuring adipose tissue IL-6 release. Nevertheless, had the resources and time been available, an investigation into the effects of diet-induced obesity on adipose tissue IL-6 release would be desirable.

Studies on IL-6 release from human adipose tissue have focused primarily on differences that occur as a result of obesity. As with the murine data presented here, human obesity is characterised by an increase in circulating IL-6, with omental adipose tissue depots releasing significantly more IL-6 than does subcutaneous tissue [236]. There is an absence of data on depot-specific differences in adipose tissue from lean humans, but if consistent with the murine data, they suggest a functional change in adipose tissue as the organism becomes obese. The increased basal IL-6 release from omental adipose tissue from obese animals compared with lean indicates that the physiology of this tissue is affected by obesity. Subcutaneous adipose tissue mass is also greater in obese individuals but, unlike omental tissue, releases similar levels of IL-6 per gram of tissue as does tissue from lean animals. The real-time rt-PCR data from the adipose tissue further indicate that constitutive release of IL-6 from adipose tissue is greater from omental depots in the obese animal than from subcutaneous depots, and that there is no such difference between the depots in lean mice. This increase in IL-6 release from omental tissue in obesity might have serious implications for the risk of obesity-related diseases, such as insulin resistance, coronary heart disease (CHD) and diabetes, all of which are correlated with visceral adiposity and increased circulating levels of IL-6.

One interesting observation concerns the rate of IL-6 release. The hourly rate of release of IL-6 from visceral tissue is similar during both the 5 h and 24 h incubation periods, suggesting that visceral tissue can release appreciable quantities of IL-6 over a relatively long period of time

in the absence of stimulation. (Notably, visceral tissue from obese animals was releasing almost double the amount of IL-6 per hour than was the tissue from lean animals. Despite not having data for 5 h release from obese tissue, because of a lack of access to the animals, the data indicate that obese tissue rate-of-release is greater than that of lean.) By contrast, the rate of subcutaneous tissue IL-6 release is lower over 24 h (from lean animals) than it is from visceral tissue. Although this could be the result of a lower release per hour, it could also derive from a reduction in release during the time course; that is, IL-6 release would initially be similar (for example, during the first 5 h), but would then decrease. In contrast to the visceral adipose tissue, therefore, subcutaneous depots do not appear to be able to continue releasing IL-6 at a high rate for long periods of time, further indicating a physiological difference between the two adipose tissue sources. However, because no assessment was made of the viability of the subcutaneous tissue after 24 h *ex vivo*, it is possible that the accumulation rate slowed as a result of cell death. To ensure that this was not the reason for the reduction in IL-6-release rate, an assay of cell viability, such as ^{125}I-insulin binding, or simpler methods, such as cell counting, cell size or trypan-blue exclusion [313], should have been carried out after 24 h in culture. Previous reports have suggested that explanted adipocytes are fully functional for up to 72 h [313], but this cannot be assumed in this case. Hourly (or similarly frequent) sampling of the supernatants would also be a valuable measurement of rate of release.

Furthermore, the stability of IL-6 is also important. Two general mechanisms should be considered: the breakdown of released IL-6 in the supernatant, and the reuptake and degradation of IL-6 by adipocytes (and other cells within the adipose tissue). The former might be a factor in the 24 h cultures; it is unlikely that there is a significant effect over 5 h, because IL-6 is stable for at least 6 h in the absence of blood cells [314] (although macrophages are likely to be present in the adipose tissue, as discussed below). Moreover, there were still significant quantities of measurable IL-6 in the supernatant from obesity-derived visceral adipose

tissue, which contains more macrophages than subcutaneous tissue [23]. The latter possibility, the re-uptake of IL-6 by adipocytes, could also be occurring. Adipocytes are known to express the IL-6 receptor system [267], and data presented in Chapters 4 and 5 describe functional effects of IL-6 on the adipocyte, indicating that IL-6 is likely to be internalised and degraded following its binding to the cell surface. This, however, is unavoidable, although frequent (e.g. hourly) sampling of the supernatant (replacing the removed media with an equivalent volume of fresh media to avoid concentrating the supernatant) during the incubation might represent a reasonable method to obtain continuous rate of release data.Of course, IL-6 release is not the only function of adipose tissue that changes in obesity. Insulin resistance is one of the best-studied disorders that is related to excess weight, and one which, with other markers of the metabolic syndrome, is closely related to fat-cell size and distribution [30, 315, 316]. Although it might seem obvious, adipocytes in obese individuals are larger than those in the lean [30], but it is important to differentiate this observation from obesity merely being due to an increase in the number of adipocytes within the depot. If obesity were characterised solely by an increase in the number of adipocytes, it would be difficult to explain how visceral adipose tissue could behave differently in the obese than in lean individuals. Changes to adipocyte size and volume are known to affect the release of adipocyte-derived factors. For example, leptin expression is proportional to the size of the lipid vacuole within the adipocyte [317], and circulating adiponectin levels are inversely related to fat mass [318, 319]. It seems likely, therefore, that IL-6 release is similarly controlled, with more secreted from omental adipose tissue in the obese state. Thorough investigations into the physiology of the hypertrophic adipocytes and adipose tissue, based upon the data collected concerning leptin, adiponectin and now IL-6, are likely to reveal the 'obese' adipocytes and tissues as functionally deviant for a number of mechanisms, some of which will result in an obesity-related disease phenotype. Furthermore, the infiltration of macrophages into adipose tissue during obesity [23] and/or the conversion of adipocytes into

macrophage-like cells [24] might explain the increased response to stimuli in the adipose tissue of the obese animals.

The reports by Weisberg *et al.* [23], Xu *et al.* [320] and Charriere *et al.* [24] raise interesting points concerning the source of adipose-tissue-derived IL-6. The two former papers describe the infiltration of adipose tissue, especially in the obese, by macrophages, and the latter the conversion of adipocytes into macrophage-like cells in obesity. Although unstimulated macrophages do not release IL-6 [235, 256], it is probable that the macrophages that have infiltrated adipose tissue are stimulated, and therefore can release IL-6. Furthermore, the expression of IL-6 was detected in both the macrophage and stromal vascular fractions, although the largest portion was expressed by adipocytes [23]. Nevertheless, a significant non-adipocyte release of IL-6 from adipose tissue has been reported [321]. Therefore, it is probable that a large portion of the IL-6 that was measured in the supernatants in this study was not specifically adipocyte derived. However, adipocyte-derived IL-6 is not insignificant; isolated adipocytes are known to release IL-6 [256, 267], and IL-1β does not affect the release of IL-6 from macrophages [235, 322]. This indicates that the IL-1β-induced release of IL-6 that was reported here was from adipocyte (or the unmeasured stromal vascular fraction). Unfortunately, time constraints prevented the isolation of adipocytes from adipose tissue to provide a definitive answer to the comparative release from each fraction in this study, and comparisons with the release from 3T3 cells is not a reasonable measure because of the relatively low level of IL-6 from cell lines [256]. Nevertheless, although adipocytes themselves are not the only source of IL-6 in adipose tissue, they do contribute to the release, especially stimulated release.

The release of IL-6 from adipocytes differs from numerous other adipokines in one important respect. Factors such as leptin [287, 302], adiponectin [187] and LPL [302, 323] are stored in secretory vesicles prior to release. The release of these molecules occurs following an external stimulus, for example insulin binding, inducing exocytosis. IL-6, however, does not appear to be stored within the adipocyte before

secretion. Stored adipokines are, at least partially, insensitive to cycloheximide treatment; sufficient product is present within the cell that further synthesis is not required for release following stimulation. IL-6 release was acutely sensitive to cycloheximide, even in the presence of stimulation by IL-1β, indicating a requirement for *de novo* production. Because the levels of IL-6 that were measured in the supernatant were barely above the background limits for the assay, it is highly unlikely that significant IL-6 was present intracellularly. Constitutive release of IL-6 is further implied by the relationship between the 5 h and 24 h incubations of C57BL6/J-derived adipose tissue. Basal release of IL-6 over 5 h is approximately one-fifth that of the 24 h release, indicating a constant and steady release. It seems, therefore, that IL-6 is constitutively expressed and released from murine adipose tissue, as it is in humans [235]. The intracellular pathway of IL-6 secretion, given that it does not correspond to those of other adipokines, needs to be determined.

Just as intriguing as the fact that basal secretion of IL-6 differs in the lean and the obese is the observation that obese visceral adipose tissue is also more responsive to stimulated IL-6 release. Omental fat from obese mice releases greater levels of IL-6 following incubation with the β3-adrenoceptor agonist CL316243. Omental tissue from lean animals and subcutaneous tissue from either lean or obese mice are unresponsive to CL316243. This discrepancy implies that omental tissue in obesity contains a greater number of, or more active, β3-adrenoceptors. However, previous studies have indicated a significant reduction in β3-adrenoceptors expression in $ob^{-/-}$ adipocytes compared with controls [324, 325]. Additionally, C57BL6/J mice fed high-fat diets to induce obesity, and other genetically obese strains also have reduced β3-adrenoceptor expression [326, 327]. These studies also described a reduction in function, as well as expression, of the receptors, which makes the increase in IL-6 secretion observed in these experiments when $ob^{-/-}$ visceral adipose tissue was treated with CL316243 difficult to explain. The changes in expression that were observed by Collins *et al.*

[326] and Breslow *et al.* [325] were on epididymal and/or perirenal fat pads: depots included in the omental tissue used in this study. One final observation from these previous studies that is of relevance here is the fact that subcutaneous adipose tissue expression of β3-adrenoceptor was identical to that of perirenal adipose tissue in lean animals (with both lower than that of epididymal), and diet-induced obesity reduced expression in all three depots to similar levels [326]. Following these findings, one might predict that treatment with a β3-specific agonist would have less effect on adipose tissue from obese animals compared with lean, with no significant difference between subcutaneous and visceral depots. Unfortunately the data obtained here does not enable a conclusive explanation of the mechanism of increased IL-6 release. One possibility is that, despite being classed as a β3-specific agonist, CL316243 (as with other adrenoceptor agonists) might also affect other β-adrenoceptors. Therefore, with fewer β3-adrenoceptors present, stimulation could be through β2-adrenoceptor, an adrenoceptor that is increased in the obese state [328]. Further studies are required to identify the precise mechanisms occurring in β-adrenoceptor-stimulated IL-6 release, possibly starting with measurement of β2-adrenoceptor (clenbuterol) agonist effects.

In comparison with CL316243, IL-1β was a more potent effector of IL-6 release from adipose tissue, with visceral tissue secreting more IL-6 (independent of the mass of the animal), whether incubated for 5 h or 24 h. Subcutaneous adipose tissue had no statistically significant response to IL-1β, barring those from obese sources, but the data reported here spans a wide range. Human subcutaneous adipocytes have been shown to respond to IL-1β *in vitro* [235], and, in light of the data presented here, murine adipocytes might be expected to respond similarly.

Having established that adipose tissue constitutively releases IL-6 in the basal state, and that this release can be upregulated by a variety of signals, it is worth comparing this release to that from other tissues. The

data obtained from the liver samples show that minimal amounts of IL-6 are released from hepatocytes in the absence of stimulation. Monocytes and macrophages are able to release IL-6 following stimulation with IL-1β, but not basally [235]. Furthermore, skeletal muscle does not release IL-6 in the absence of a stimulant, such as exercise [232]. Therefore, of the major IL-6-releasing organs and tissues, only adipose tissue, and particularly visceral depots, releases IL-6 constitutively and in significant amounts in the absence of a stimulatory signal.

The experiments presented here provide a better understanding of the mechanisms of the release of IL-6 from adipose tissue. As in humans, murine adipose tissue releases IL-6 constitutively [235], and this is increased in obesity. Unlike numerous other adipokines, IL-6 is not stored within the adipocyte until secretion is stimulated, but is synthesised *de novo*. The immune modulator IL-1β is a powerful stimulator of IL-6 release from adipose tissue, but adrenoceptor stimulation is only effective in visceral tissue from obese animals. The importance of adipose-tissue-derived IL-6 as an autocrine/paracrine signal is discussed further in Chapters 4 and 5.

Chapter 4 – Adipogenesis

Introduction

The total mass of adipose tissue is determined by both the number of adipocytes and the volume of these cells within the tissue. Under normal conditions, the number of adipocytes is regulated by the formation of new adipocytes through the differentiation of preadipocyte precursors (adipogenesis, Figure 1.3) and apoptosis of existing adipocytes. The volume of the cells, itself dependent upon lipid content, is regulated by metabolic processes such as lipogenesis and lipolysis. A number of cytokines and adipokines have important roles in the adipogenic and apoptotic regulation of adipocytes. However, whether IL-6 can alter adipocyte numbers, by the regulation of adipogenesis and apoptosis and/or adipocyte size, through effects on glucose uptake, lipogenesis and lipolysis, is not known.

The best-characterised adipokine affecting these adipocyte functions is TNFα, a potent anti-adipogenic factor. The incubation of adipocytes with TNFα reverses differentiation, probably owing to an inhibition of the adipogenic mediator PPARγ [99, 171]. TNFα also initiates apoptosis in mature adipocytes [93]. By contrast, the majority of studies using leptin have reported no significant autocrine/paracrine effects on adipocyte differentiation [126] (although a pro-adipogenic effect has been reported in rodents [329]). However, leptin has been implicated in the apoptosis of adipocytes [311], although this is likely to be mediated through indirect central effects rather than an autocrine/paracrine response [127, 330]. Resistin has been reported to inhibit adipocyte differentiation [175], although there are very little data available concerning this adipokine and its molecular function.

Previous studies have shown that IL-6 inhibits the expression of PPARγ [265], and inhibits adipocyte differentiation under certain conditions in bone marrow stroma [331]. However, IL-6 has also been reported to increase adipogenesis at the expense of osteoblastogenesis in SAMP6 mice [332], and other members of the class I cytokine family

have been reported to increase adipogenesis via the MAPK cascade [333], which can also be activated by IL-6. IL-6 is also a growth and differentiation factor for a number of other cell types [334, 335]. The direct role of IL-6 in adipogenesis has not been studied, however.

Early changes in cellular morphology during adipogenesis, with a rounding of cells, can be observed microscopically. Lipid droplets are also visible within 72 h after the induction of adipogenesis, and these lipid deposits accumulate until it accounts for over 80% of the cellular contents. These morphological and biochemical changes are accompanied by changes in gene expression. Leptin release from these cells also increases, and its secretion is directly related to the lipid content of the cells [34, 35].

The initial experiments described in this chapter were loosely based on the studies of TNFα and adipocyte differentiation. With both IL-6 and TNFα being pro-inflammatory cytokines, and TNFα potently inhibiting adipogenesis, the hypothesis for the research was that IL-6 would have a similar effect.

This chapter will investigate the effect of IL-6 on adipocyte differentiation using the conversion of 3T3-F442A preadipocytes to mature adipocytes as an *in vitro* model of adipogenesis. Adipogenesis, initiated using a number of frequently cited methods, was monitored by investigating changes in cellular morphology, lipid accumulation, gene expression and leptin release.

Methods

The effects of IL-6 on *in vitro* adipogenesis were measured using the preadipocyte cell line 3T3-F442A, which can differentiate in the absence of thiazolidinedione stimulation, and release appreciable levels of leptin when mature. By contrast, 3T3-L1 adipocytes are more difficult to differentiate, frequently requiring the addition of a thiazolidinedione to ensure adipogenesis. Time constraints precluded the investigation of other cell types and sources, such as cell lines of uncommitted stem cells that can differentiate into adipocytes, or primary preadipocytes.

3T3-F442A cells were seeded onto 6-well plates at a concentration of 6 x 10^4 cells/well in DMEM/10% BCS (supplemented, as was all cell-culture media, with 1% penicillin/streptomycin). Following a 24 h incubation (at 37°C, 5% CO_2) after seeding, cells were fed with 1.5 ml/well DMEM/10% CCS and grown until the cells were approximately 90% confluent (assessed by eye using light microscopy). The cells were then differentiated using the methods described in Chapter 2. These were:

(i) DMEM/10% CCS, supplemented with dexamethasone (1 μM), IBMX (0.5 mM) and insulin (5 μg/ml).

(ii) DMEM/10% CCS, supplemented with IBMX (1 mM) and insulin (5 μg/ml).

(iii) DMEM/10% CCS, supplemented with indomethacin (125 μM) and insulin (5 μg/ml).

(iv) DMEM/10% CCS, supplemented with T_3 (2 nM) and insulin (17 nM).

Methods (i), (iii) and (iv) are based on previously reported adipogenesis techniques (see Chapters 1 and 2), whereas method (ii) was included as a novel method that excluded dexamethasone, which is a multi-functional potentiator, to test differentiation with fewer adipogenic compounds.

For methods (i), (ii) and (iii), the cells were incubated (37°C, 5% CO_2) for 72 h in 1.5 ml/well induction media (the time of addition

designated day 0), whereupon the cells were fed with fresh media with all supplements withdrawn, bar insulin. Cells were re-fed in this manner every 48 h. Media that was removed prior to feeding was retained and stored at –80°C for subsequent assay. The protocol for method (iv) differed in that T_3 was maintained in the media at every feeding. A final set of cells served as a negative control, with cells fed only DMEM/10% CCS (Figure 4.1). In addition to the methods above, each was repeated in the presence of 10 ng/ml IL-6, included as a supplement in the media at each feeding. A concentration of 10 ng/ml IL-6 was chosen because it is within the physiological concentration range of the cytokine in the adipose tissue [256] (and unpublished data). Had more time been available, a range of IL-6 concentrations would have been investigated, from ~0.1–100 ng/ml IL-6 (which is still below the concentration used in other studies [267]).

A subset of cells, treated identically to those in method (i), in the absence or presence of IL-6, was used for RNA isolation and rt-PCR. Media was removed and retained for assay, and the 6-well plates were flash frozen in liquid nitrogen and stored at –80°C prior to RNA isolation. RNA isolation, cDNA synthesis and rt-PCR were carried out as described in Chapter 2. End-point rt-PCRs were carried out for β-actin, PPARγ, C/EBPα, *ob* and adipsin. Additionally, to enable a comparison with cells treated with IL-6 for a considerably shorter time period, cells differentiated for 15 days using method (i) were treated for 48 h in Cell-gro, in the absence or presence of 10 ng/ml IL-6. The media from these cells was assayed for leptin release, and rt-PCR was carried out.

A final subset of cells was differentiated, on cover slips using method (i) in the absence or presence of IL-6, and used to assess lipid accumulation. Cells were loaded with BODIPY 493/503 and the lipid content of a random selection of cells assessed by confocal microscopy (see Chapter 2). A total of 82 randomly selected control cells and 40 IL-6-treated cells were analysed.

Data are presented as median and interquartile range, and were obtained from a minimum of six individual samples from at least two

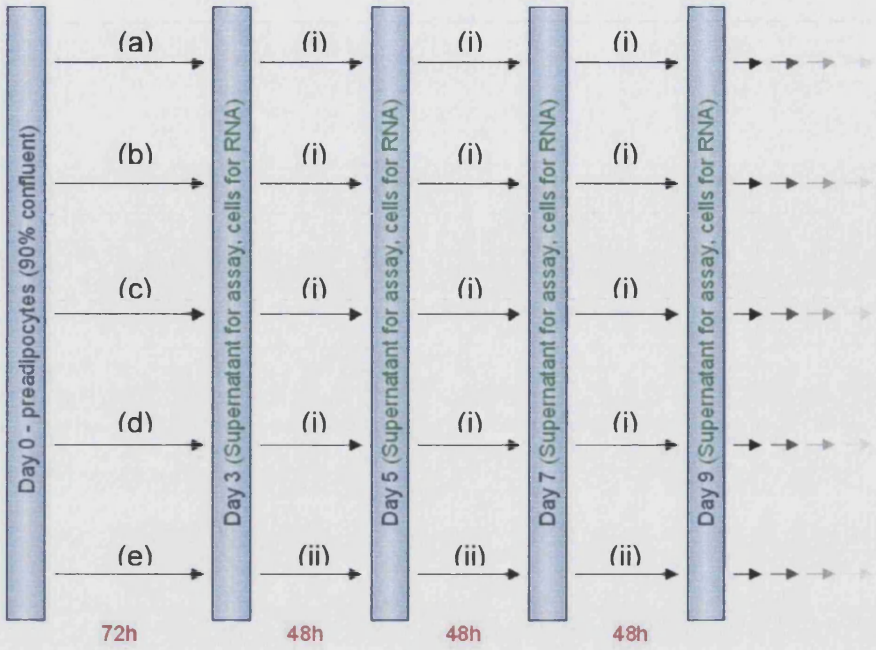

Figure 4.1 – Time line of adipogenesis cell culture.

3T3-F442A preadipocytes were treated at day 0 with: (a) dexamethasone, IBMX and insulin; (b) IBMX and insulin; (c) indomethacin and insulin; (d) T_3 and insulin; or (e) no treatment, all in the absence or presence of 10 ng/ml IL-6. After 72 h, supernatant was removed for assay (and cells for RNA extraction) and cells re-fed with: (i) insulin-supplemented media or (ii) T_3 and insulin (as day 0), with or without IL-6. This removal and re-feeding was repeated every 48 h until the cessation of the experiment (day 17)

separate experiments (*n* values refer to the number of individual wells from which measurements were obtained). Significance was calculated using the Mann-Whitney U test, with two-tailed *p* values below 0.05 considered significant. Real-time rt-PCR data are expressed as changes in expression compared with an arbitrary baseline (described for each individual message investigated), using the method described by Livak and Schmittgen [295].

Results

Differentiation assessed by microscopy

Changes in the morphology of the cells during differentiation were assessed by microscopy. Using light microscopy, it was evident that within 72 h of induction of differentiation using the dexamethasone or indomethacin containing protocols [methods (i), (ii) and (iii)], the majority (> 90%) of cells had changed from the preadipocyte spindle shape to the rounded morphology of the adipocyte. At 7–9 days post-induction of differentiation small lipid droplets were visible, which continued to expand until > 90% of the cell volume was lipid (by day 17 post-induction). The changes observed during the T_3 protocol were less pronounced; fewer cells adopted the adipocyte morphology within 72 h, lipid droplets were not visible until much later post-induction of differentiation and the lipid vacuoles were smaller. Preadipocytes that were not incubated with any of the traditional adipogenic agents did not adopt an adipocyte-like morphology or accumulate lipid, with the exception of a tiny minority of cells that underwent spontaneous differentiation.

The inclusion of 10 ng/ml IL-6 to the dexamethasone- or indomethacin-containing protocols resulted in larger lipid droplets within the cell when compared with non-IL-6-treated cells at the same time point. There was no visible effect of IL-6 on the cells that were differentiated using T_3 and insulin, or on untreated cells.

Treating adipocytes with the neutral lipid dye BODIPY 493/503 confirmed the observation that IL-6 increased the lipid content of adipocytes during differentiation with dexamethasone, IBMX and insulin (Figures 4.2 and 4.3). The mean index of BODIPY staining for cells differentiated in the absence of IL-6 was 0.49 (0.42–0.53), compared with 0.68 (0.57–0.77) for IL-6-treated cells ($p < 0.01$).

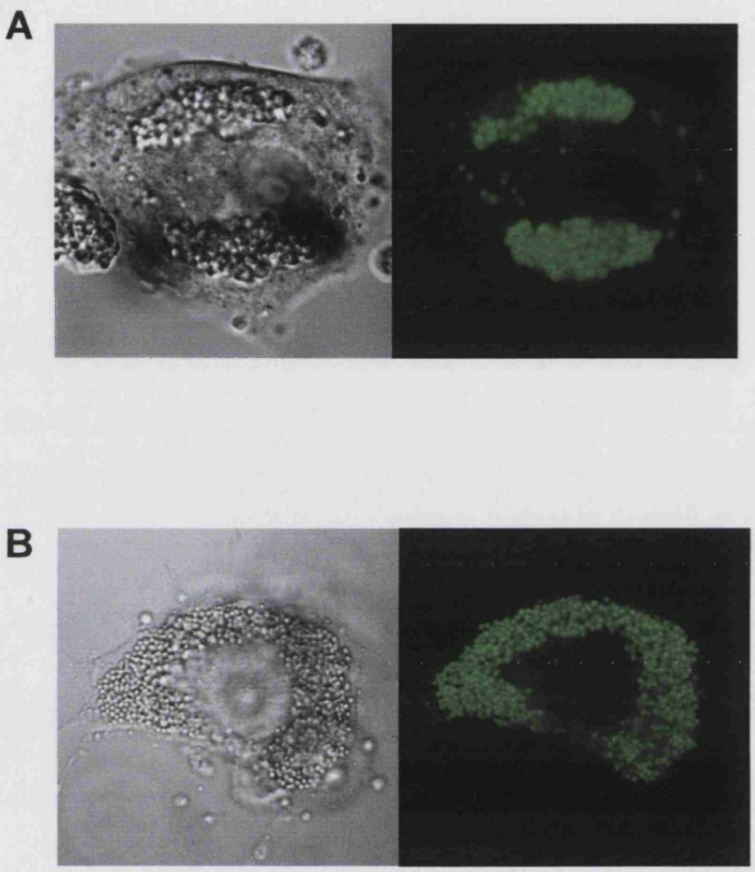

Figure 4.2 – Confocal Microscopy of cells differentiated with and without IL-6.

Representative adipocytes at 13 days post-induction, differentiated in the absence **(A)** or presence **(B)** of 10 ng/ml IL-6 using the dexamethasone, IBMX and insulin protocol. The left-hand panels show the transmitted images; right hand panels are images of the cells dyed with the neutral lipid-staining BODIPY 493/503.

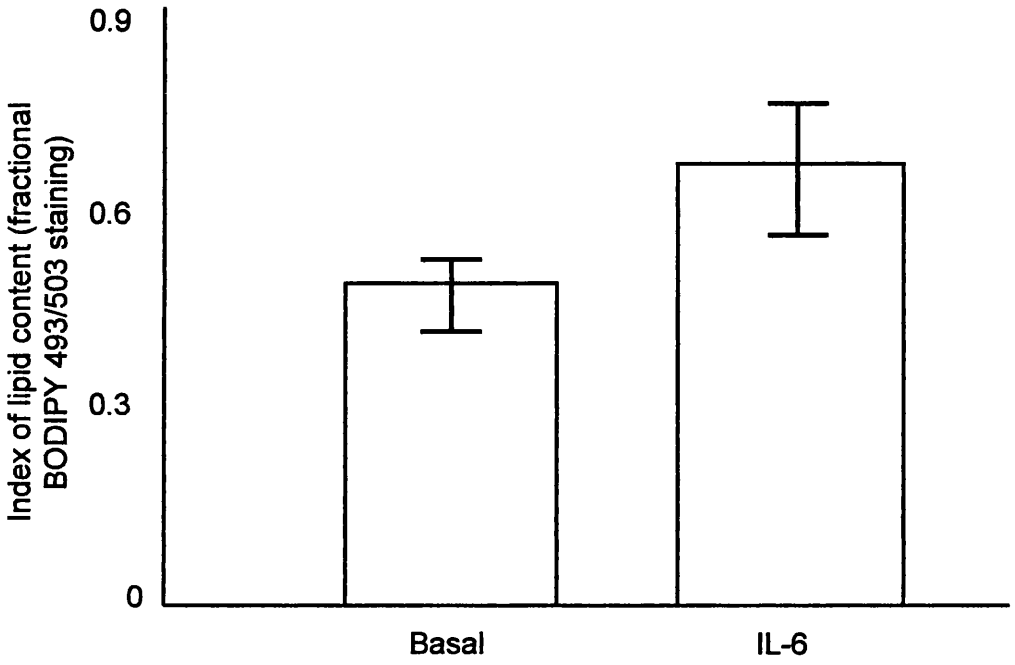

Figure 4.3 – Index of BODIPY 493/503 staining of 3T3-F442A cells differentiated in the presence or absence of IL-6.

The index of BODIPY 493/503 staining, representing cellular lipid content, of 3T3-F442A adipocytes differentiated using dexamethasone, IBMX and insulin in the absence (basal, n= 82) or presence (n= 40) of 10 ng/ml IL-6.

$p < 0.01$

Differentiation assessed by leptin release

The secretion of the adipocyte-derived protein leptin was used for an assessment of adipogenesis. Although leptin is not a marker of adipogenesis *per se*, its expression and release is related to the volume of the lipid vacuole within the cell [34], and, as such, it is a useful measure of adiposity and differentiation. Depending upon the differentiation method used, leptin accumulation in the media increased significantly 5–9 days post-induction of differentiation, compared with day 0 (Figures 4.4–4.8). Leptin levels from cells differentiated using dexamethasone, IBMX and insulin rose above preadipocyte levels nine days after induction [day 0, 25.9 (17.6–31.4) pg/ml leptin vs. 52.0 (38.3–144.6) pg/ml day 9, $p< 0.001$], whereas in the presence of IL-6 the difference was observed at day 7 [32.7 (26.6–38.1) pg/ml, $p= 0.005$]. Preadipocytes that were treated with IBMX and insulin released significantly more leptin at day 9 compared with day 0, whether IL-6 was present or not [day 0, 13.9 (13.5–14.6) pg/ml leptin vs. day 9 no IL-6, 17.7 (15.2–19.4) pg/ml, $p= 0.037$; day 9 + IL-6, 75.6 (42.6–188.4) pg/ml, $p= 0.004$]. Changes in leptin release from indomethacin-treated cells were noticeable even earlier during the time course, at day 7 in the absence of IL-6 [day 0, 22.0 (19.9–23.6) pg/ml leptin vs. 52.1 (40.2–67.9) pg/ml, $p= 0.004$] and day 5 in its presence [23.8 (23.7–24.9) pg/ml, $p= 0.025$]. Interestingly, both untreated cells and those that were treated with T_3 had significant differences in leptin levels from day 5, despite having very few differentiated cells or lipid vacuoles visible. Notably, although the differences in release were significant, the actual levels of leptin were considerably lower throughout the time-course of the experiment than those measured at comparable time points using the other methods of differentiation [T_3 day 0, 7.9 (7.5–8.1) pg/ml leptin vs.: day 5 no IL-6, 24.0 (19.7–30.0) pg/ml, $p= 0.004$; day 5 + IL-6, 19.7 (18.7–-20.7) pg/ml, $p= 0.004$; and no intervention day 0, 20.4 (18.4–22.2) pg/ml leptin vs.; day 7 no IL-6, 29.3 (26.3–35.0) pg/ml, $p= 0.025$; day 5 + IL-6, 27.5 (25.0–34.7) pg/ml, $p= 0.017$]. With the exception of untreated cells

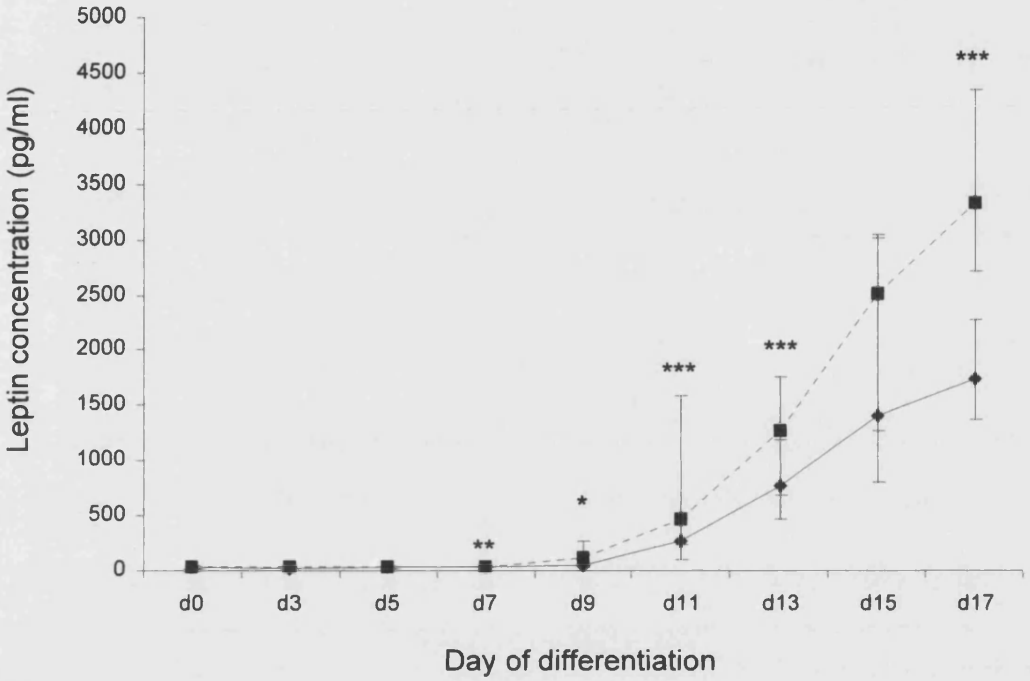

Figure 4.4 – Leptin release from 3T3-F442A cells differentiated using dexamethasone, IBMX and insulin, $^{+}/_{-}$ IL-6.

The accumulation of leptin in the media of differentiating 3T3-F442A adipocytes treated for 72 h (at day 0) with dexamethasone, IBMX and insulin in the absence (unbroken line) or presence (broken line) of 10 ng/ml recombinant murine IL-6. Data presented as median and interquartile range (n≥ 22 for each sample).

* $p< 0.001$, no IL-6 vs. d0; ** $p= 0.005$, IL-6 treated vs. d0; *** $p\leq 0.026$, IL-6 treated vs. untreated.

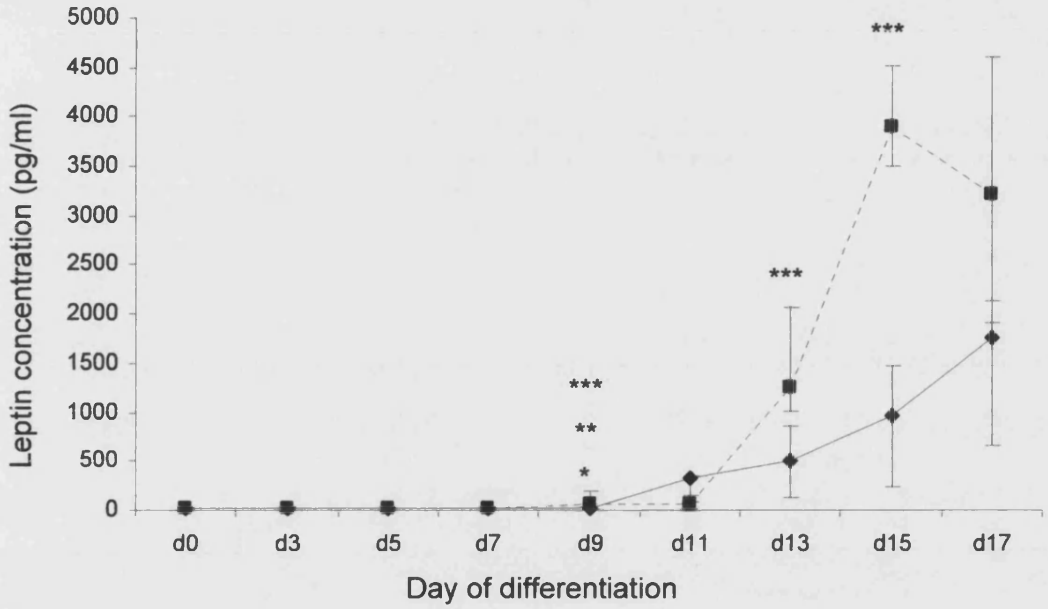

Figure 4.5 – Leptin release from 3T3-F442A cells differentiated using IBMX and insulin, $^{+}/_{-}$ IL-6.

The accumulation of leptin in the media of differentiating 3T3-F442A adipocytes treated for 72 h (at day 0) with IBMX and insulin in the absence (unbroken line) or presence (broken line) of 10 ng/ml recombinant murine IL-6. Data presented as median and interquartile range (n= 6 for each sample).

* p= 0.037, no IL-6 vs. d0; ** p= 0.004, IL-6 treated vs. d0; *** $p \leq$ 0.016, IL-6 treated vs. untreated.

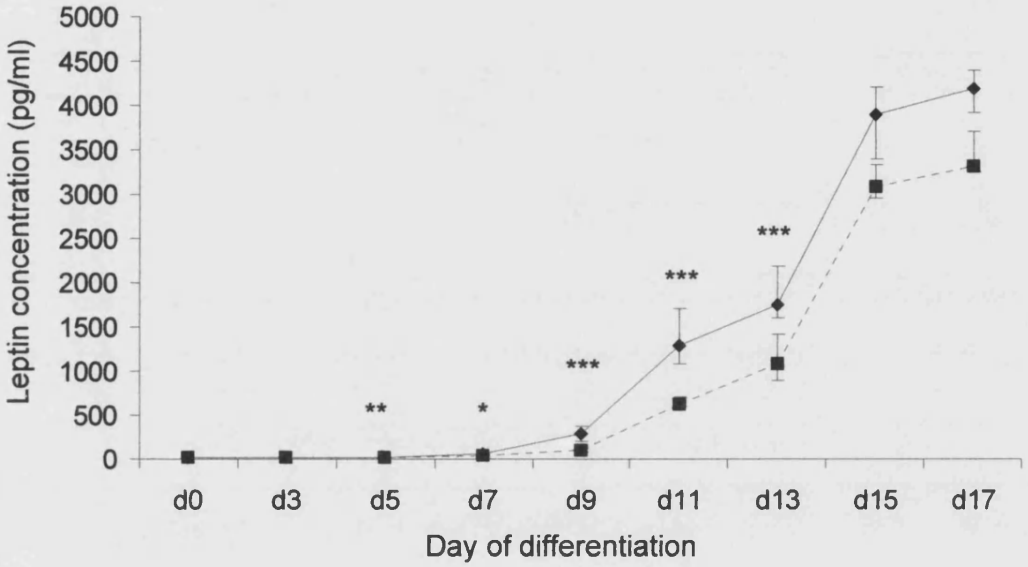

Figure 4.6 – Leptin release from 3T3-F442A cells differentiated using indomethacin and insulin, $^{+}$/- IL-6.

The accumulation of leptin in the media of differentiating 3T3-F442A adipocytes treated for 72 h (at day 0) with indomethacin and insulin in the absence (unbroken line) or presence (broken line) of 10 ng/ml recombinant murine IL-6. Data presented as median and interquartile range (n= 6 for each sample).

* p= 0.004, no IL-6 vs. d0; ** p= 0.025, IL-6 treated vs. d0; *** $p \leq$ 0.016, IL-6 treated vs. untreated.

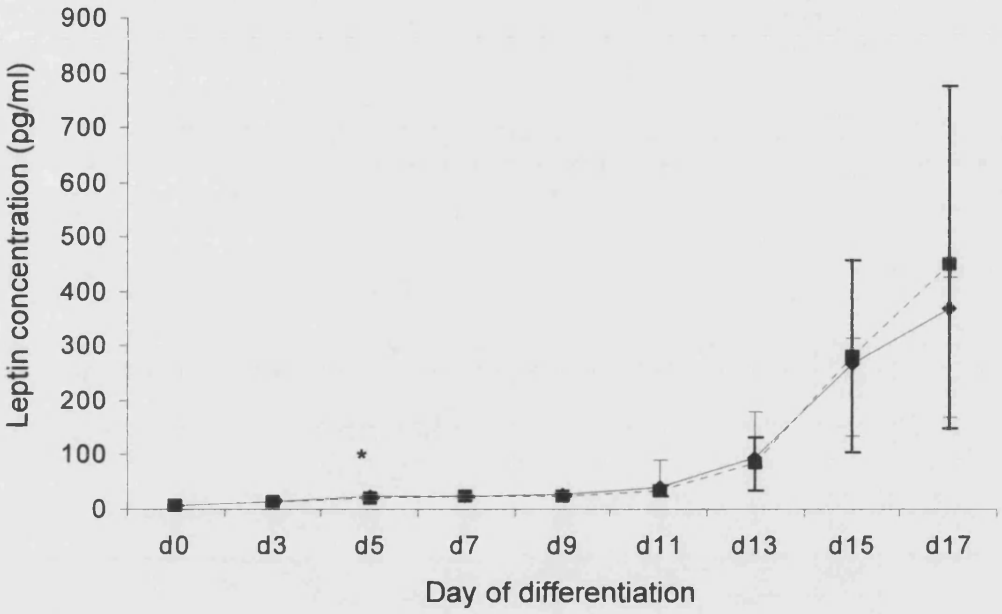

Figure 4.7 – Leptin release from 3T3-F442A cells differentiated using T_3 and insulin, $^{+}$/- IL-6.

The accumulation of leptin in the media of differentiating 3T3-F442A adipocytes treated for 72 h (at day 0) with T_3 and insulin in the absence (unbroken line) or presence (broken line) of 10 ng/ml recombinant murine IL-6. Data presented as median and interquartile range (n= 6 for each sample).

* p= 0.004, both IL-6 treated and untreated vs. d0.

Day	Dex, IBMX, ins	Dex, IBMX, ins, IL-6	IBMX, ins	IBMX, ins, IL-6	Indo, ins	Indo, ins, IL-6	T_3	T_3, IL-6
0	25.9 17.6–31.4	25.9 17.6–31.4	13.9 13.5–14.6	13.9 13.5–14.6	22.4 19.9–23.6	22.4 19.9–23.6	7.9 7.5–8.1	7.9 7.5–8.1
3	23.0 19.2–29.6	28.1 18.9–33.1	19.1 18.7–20.9	19.8 15.2–33.0	23.6 20.4–25.1	22.3 20.1–22.9	12.3 14.8–15.5	15.2 14.8–15.5
5	27.7 21.1–34.3	31.6 22.0–41.1	14.8 13.7–15.6	12.2 12.0–15.9	21.8 21.2–22.3	23.8 23.7–24.9	24.0 19.7–30.0	19.7 18.7–20.7
7	30.7 24.4–38.6	32.7 26.6–38.1	15.0 12.6–16.1	18.4 15.9–19.5	52.1 40.2–67.9	34.5 29.7–37.9	22.9 22.6–23.7	22.3 20.4–23.4
9	52.0 38.3–144.5	111.9 40.1–266.8	17.7 15.2–19.4	75.6 42.6–188.4	294.5 209.6–368.7	106.1 127.7–89.5	26.1 22.9–30.7	24.4 22.3–27.3
11	260.3 102.2–460.1	466.8 231.5– 1593.4	324.1 156.6–357.0	57.5 30.0–91.2	1283.4 1086.0– 1708.4	632.2 601.0–714.2	42.1 38.5–90.8	35.4 24.9–45.6
13	772.8 474.4– 1185.9	1264.1 679.5– 1749.5	513.8 136.7–854.5	1255.2 1006.0– 2081.2	1746.7 1594.4– 2179.6	1084.3 904.5– 1412.6	93.9 78.4–179.7	83.6 32.6–130.6
15	1398.8 798.7– 3014.1	2512.4 1278.7– 3036.5	961.4 242.6– 1470.7	3900.0 3504.0– 4519.0	3894.1 3404.1– 4215.6	3090.2 2962.2– 3328.5	266.9 135.7–314.7	281.9 105.9–457.9
17	1744.1 1369.5– 2276.5	3319.6 2716.6– 4340.2	1762.8 671.3– 2127.7	3224.9 1922.8– 4598.7	4179.3 3917.4– 4394.4	3310.9 3255.7– 3712.3	367.6 168.2–247.0	451.3 150.2–775.8

Figure 4.8. Comparison of leptin release into the supernatant from 3T3-F442A cells differentiated using various methods.

The accumulation of leptin (in pg/ml) into the supernatant during the differentiation of 3T3-F442A cells with: dexamethasone (dex), IBMX and insulin (ins); IBMX and insulin; indomethacin (indo) and insulin; or T_3 and insulin. Day indicates day of differentiation.

(in the presence or absence of IL-6), leptin release continued to rise as the cells differentiated further, until the cessation of the experiment (17 days post-induction).

The effect of the inclusion of 10 ng/ml IL-6 in the induction media depended on the differentiation method used. Levels of leptin in the media were greater in cells that were differentiated with dexamethasone, IBMX and insulin, or IBMX and insulin in the presence of IL-6 when compared with equivalent cells without IL-6. This was evident from day 11 post-induction in the former method [no IL-6, 260.3 (102.2–460.1) pg/ml leptin vs. + IL-6, 466.8 (231.5–1593.4) pg/ml, p= 0.016] and from day 9 in the latter [no IL-6, 17.7 (15.2–19.4) pg/ml leptin vs. + IL-6, 75.6 (42.6–188.4) pg/ml, p= 0.004]. This trend continued until the end of the time-course, with the exception of day 15 post-induction in the dexamethasone-based method [no IL-6, 1398.8 (798.7–3014.1) pg/ml leptin vs. + IL-6, 2512.4 (1278.7–3036.5) pg/ml, p= 0.137] and day 17 in the IBMX and insulin method [no IL-6, 1762.8 (671.3–2127.7) pg/ml leptin vs. + IL-6, 3224.9 (1922.9–4598.7) pg/ml, p= 0.201]. The release of leptin during differentiation using indomethacin and insulin appeared to have the opposite effect when IL-6 was present, with IL-6-treated cells releasing less leptin [day 9 no IL-6, 294.5 (209.6–268.7) pg/ml leptin vs. day 9 + IL-6, 106.1 (89.5–127.7) pg/ml, p= 0.004]. This trend continued at days 11 and 13 post-induction. However, at both day 15 and day 17 post-induction, the difference in leptin release between the two treatments was not significant [day 15 no IL-6, 3894.1 (3404.1–4215.6) pg/ml leptin vs. day 15 + IL-6, 3090.2 (2962.2–3328.5) pg/ml, p= 0.109; and day 17 no IL-6, 4179.3 (3917.4–4394.4) pg/ml vs. day 17 + IL-6, 3310.9 (3255.7–3712.3) pg/ml, p= 0.078]. Leptin release during differentiation by T_3 and insulin was unaffected by the presence of IL-6. Untreated adipocytes release very little leptin [a peak of 56.9 (49.9–62.2) pg/ml at 13 days post-induction], but IL-6-treated cells appeared to release more leptin at day 11 post-induction, and less at day 13 [day 11 no IL-6, 43.0 (41.6–44.0) pg/ml leptin vs. day 11 + IL-6, 49.3 (46.1–52.2) pg/ml, p= 0.025; and day

13 no IL-6, 56.9 (49.9–61.2) pg/ml vs. day 13 + IL-6, 31.6 (29.1–44.8) pg/ml, p= 0.025].

Comparisons of leptin release from the different methods tended to mirror the differences observed by light microscopy. In the absence of IL-6, indomethacin-treated cells released the greatest amount of leptin at every time point from day 7 post-induction onwards. There was little discernable difference between the two methods using IBMX, both of which, in addition to the indomethacin method, tended to release more leptin than either the untreated or T_3-treated cells. Despite releasing lower levels of leptin than the other methods, cells differentiated using T_3 still released significantly more leptin than untreated cells. Among the IL-6-treated cells, the leptin release from the dexamethasone, IBMX and insulin, the IBMX and insulin and the indomethacin and insulin protocols were similar, and all significantly higher than untreated or T_3 and insulin treated cells.

Gene expression during adipogenesis

End-point rt-PCR was used to assess the expression of PPARγ, C/EBPα, *ob*, adipsin and IL-6 during differentiation that was induced with dexamethasone, IBMX and insulin. β-Actin expression was used as a control. The correct number of cycles was determined to ensure that the rt-PCR was stopped during the linear range of replication (cycle numbers are shown in Figure 2.1). The level of β-actin expression remained the same throughout the differentiation process (Figure 4.9). The increase in expression of both adipsin (an early adipocyte marker) and leptin as the differentiation time-course continued indicated that the cells were becoming mature adipocytes. IL-6 appeared to have no effect on adipsin expression, but there seemed to be a slight reduction in *ob* expression at day 17 post-induction, in the presence of IL-6.

PPARγ expression increased from the preadipocyte state to 17 days post-induction. Conversely, IL-6 expression appeared to become slightly lower. Investigation of the two major differentiation factors PPARγ

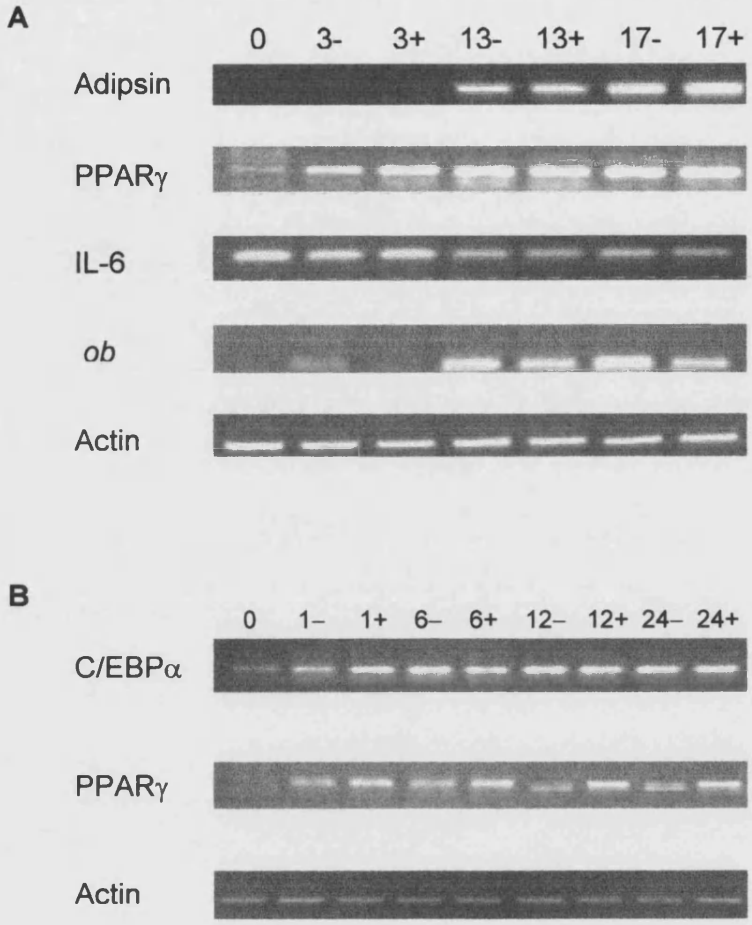

Figure 4.9 – End-point rt-PCR of adipocyte genes during differentiation.

The expression of adipocyte gene-products days **(A)** or hours **(B)** following the induction of differentiation, in the absence (–) or presence (+) of 10 ng/ml IL-6.

and C/EBPα in the hours immediately following treatment with the differentiation agents showed an immediate upregulation of both genes (within 1 h). IL-6 appeared to have no effect on the expression of C/EBPα, but increased the transcription of PPARγ at 12 h and 24 h post-induction of differentiation, and possibly also at three days post-induction.

Owing to the difficulty in quantitatively assessing the data that were produced by end-point rt-PCR, a semi-quantitative method of rt-PCR was used. Therefore, to validate the observations made using end-point rt-PCR, the adipogenesis experiments were repeated with real-time rt-PCR. Specific primers were designed for PPARγ, C/EBPα, *ob* and adipsin, with GAPDH as a control. During the first 72 h of adipogenesis, PPARγ expression rose rapidly, reaching a peak at 48 h of 11.2 (9.0–19.0) times that of the preadipocyte (Figure 4.10). This expression was subsequently reduced, but remained greater than the preadipocyte level (all samples were significantly greater than the preadipocyte expression, $p= 0.004$). Incubation with IL-6 had no significant effect on PPARγ expression during the initial 48 h, but resulted in reduced expression at 72 h post-induction [expression compared with preadipocyte (day 0); no IL-6, 4.8 (4.6–5.8) fold vs. +IL-6, 3.0 (2.9–3.2) fold, $p= 0.004$]. The rise in transcription of C/EBPα was less marked than that of PPARγ (Figure 4.11), reaching a peak of 2.3 (0.8–2.3) times greater expression than the preadipocyte at 48 h post-induction, although this rise was not statistically significant ($p= 0.055$), whereas the 1.4 (1.4–1.7) fold increase in expression at 72 h was ($p= 0.025$). The inclusion of IL-6 in the induction media caused a significant inhibition of C/EBPα expression during the first 24 h [+IL-6, 0.7 (0.6–0.7) fold expression compared with preadipocyte (day 0), $p= 0.037$; vs. no IL-6, 1.5 (1.2–17.1) fold, $p= 0.006$]. IL-6 had no effect on C/EBPα expression at 48 h or 72 h post-induction. The expression of adipsin (Figure 4.12) increased throughout adipogenesis up to and including day 17 post-induction [7.5 (6.7–8.7) fold increase in mRNA compared with preadipocyte, $p= 0.004$]. It was at this time point that the IL-6-treated cells had a significantly lower expression

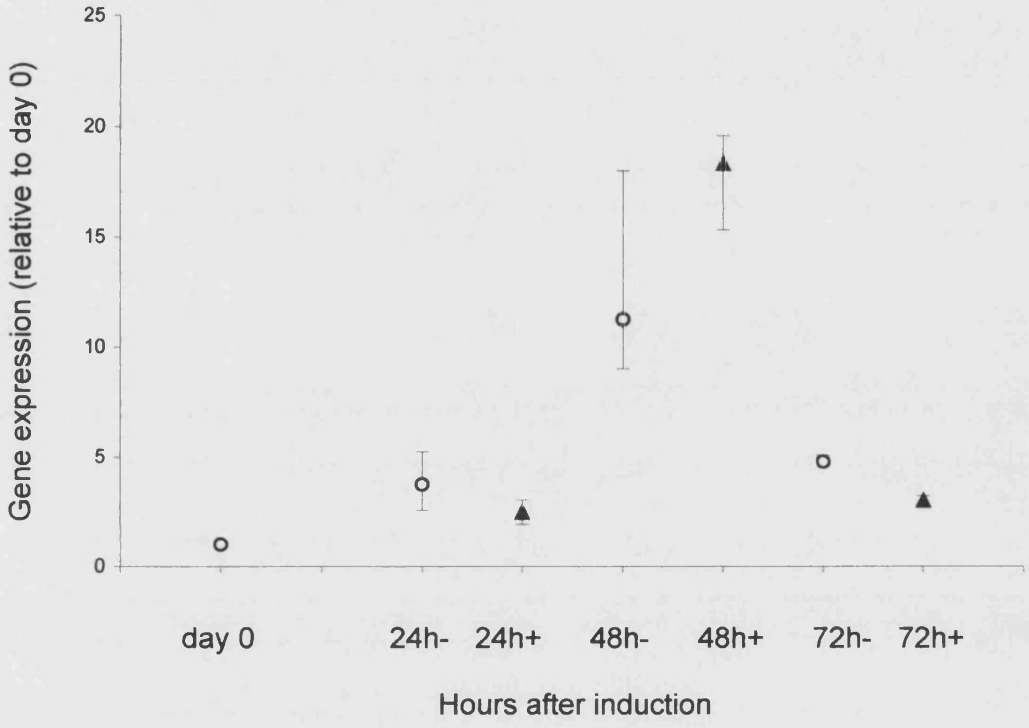

Figure 4.10 – Real-time rt-PCR analysis of PPARγ expression during adipogenesis.

The semi-quantative expression of PPARγ during adipogenesis stimulated with dexamethasone, IBMX and insulin in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels and displayed as median (interquartile range) fold-increase compared to preadipocyte levels (day 0), which are valued (arbitrarily) at 1.

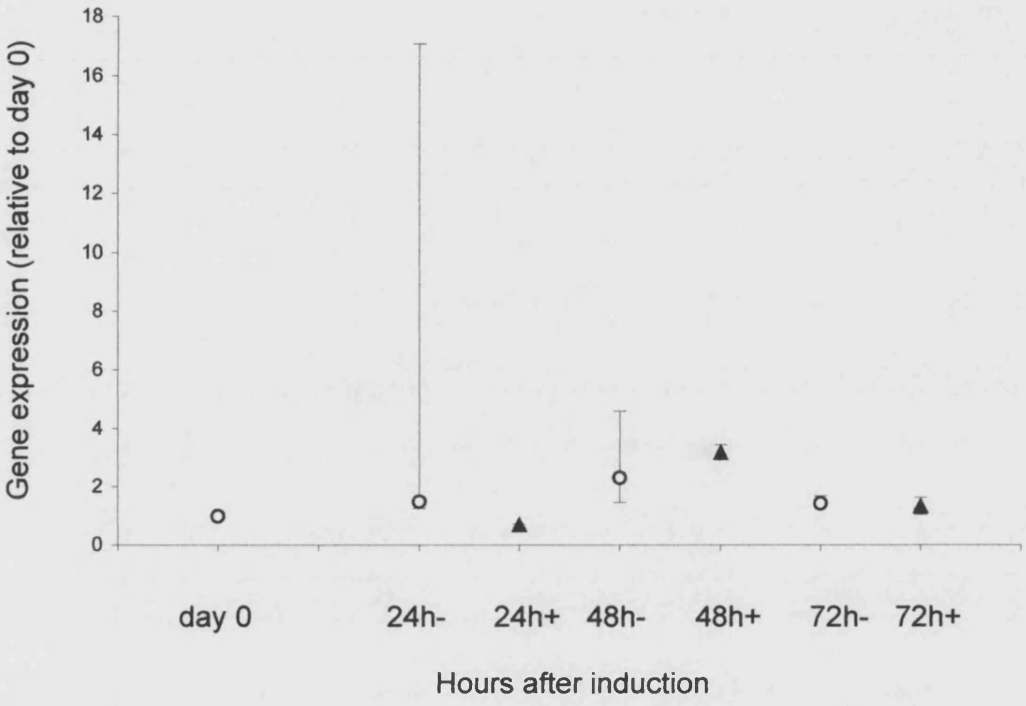

Figure 4.11 – Real-time rt-PCR analysis of C/EBPα expression during adipogenesis.

The semi-quantative expression of C/EBPα during adipogenesis stimulated with dexamethasone, IBMX and insulin in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels and displayed as median (interquartile range) fold-increase compared with preadipocyte levels (day 0), which are valued (arbitrarily) at 1.

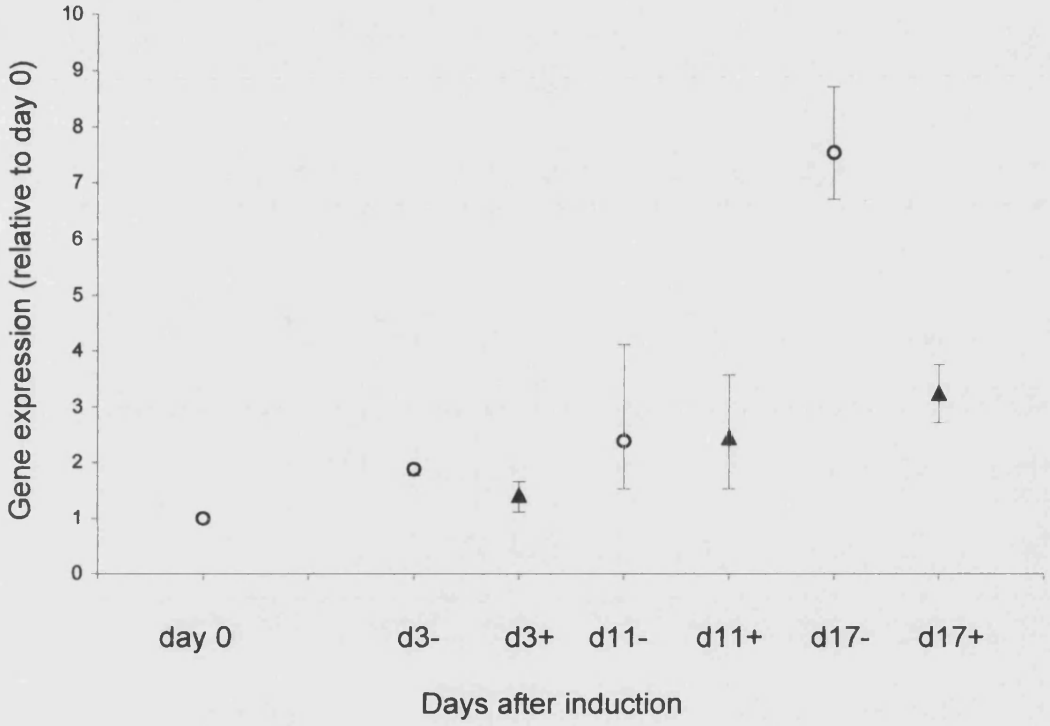

Figure 4.12 – Real-time rt-PCR analysis of adipsin expression during adipogenesis.

The semi-quantative expression of adipsin during adipogenesis stimulated with dexamethasone, IBMX and insulin in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels and displayed as median (interquartile range) fold-increase compared with preadipocyte levels (day 0), which are valued (arbitrarily) at 1.

of adipsin compared with those cells that were differentiated without IL-6 [p= 0.006; expression of adipsin in IL-6 treated cells, compared with preadipocyte, 3.2 (2.7–3.8) fold greater]. Comparison of the expression of *ob*, the leptin gene, in cells treated with or without IL-6 during differentiation indicated that, in spite of the increase in leptin release that was observed in the presence of IL-6, transcription was not affected (Figure 4.13). No significant difference occurred at 11-, 15- or 17-days post-induction of differentiation.

Short-term IL-6 incubation with adipocytes

In order to investigate the acute effects of IL-6 on leptin expression and release in adipocytes, 3T3-F442A preadipocytes were differentiated using dexamethasone, IBMX and insulin. At 15-days post-induction (when they were mature adipocytes), the cells were treated with Cell-gro in the absence or presence of 10 ng/ml IL-6, for up to 48 h. There was no significant difference in leptin release over 48 h between the IL-6 treated [2531.2 (2265–3326) pg/ml] and untreated [2392.3 (2314–2925) pg/ml, p= 0.827] cells. This was matched by the expression of *ob*, measured by real-time rt-PCR [IL-6 treated expression was 1.1 (0.6–1.6) fold that of untreated cells, p= 0.873].

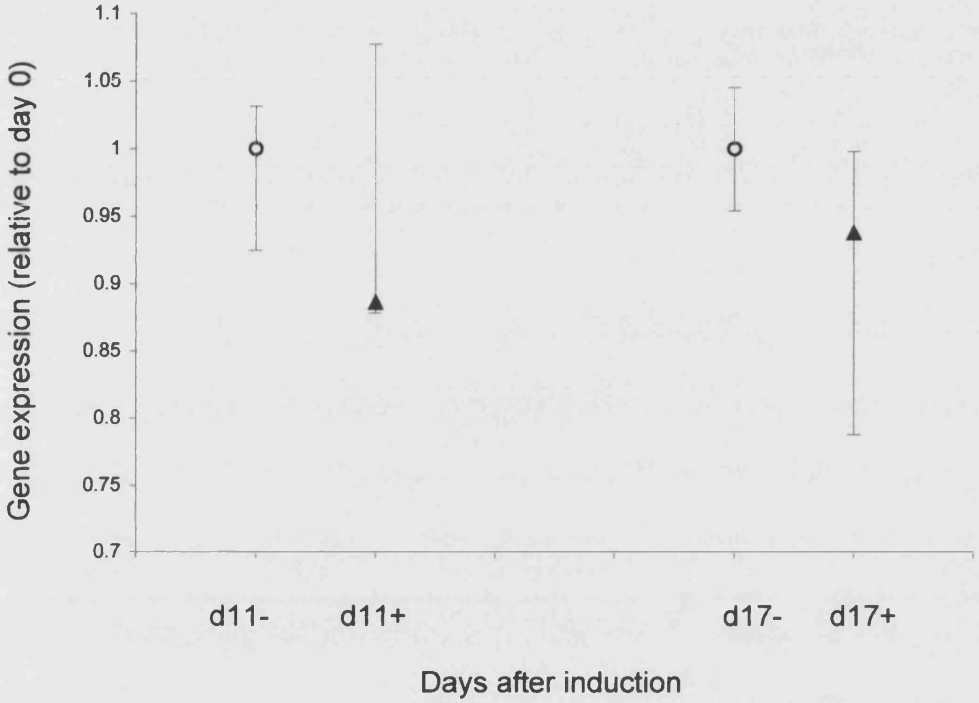

Figure 4.13 – Real-time rt-PCR analysis of *ob* expression during adipogenesis.

The semi-quantative expression of *ob* during adipogenesis stimulated with dexamethasone, IBMX and insulin in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels and displayed as median (interquartile range) fold-change in expression with IL-6 treatment compared to the corresponding untreated levels (day 0), which are valued (arbitrarily) at 1.

Discussion

Even the use of the most basic technique available, light microscopy, indicated that there was a clear, discernable difference between the adipocytes that were differentiated in the presence of IL-6 and those that were not. The initial observation that increased cell size and lipid-vacuole volume occurred in the presence of IL-6 was confirmed by using leptin as an adipocyte marker, dramatically disproving the original hypothesis, which predicted the opposite. Leptin is produced almost exclusively by adipocytes, and circulating levels are strongly correlated with indices of obesity, such as BMI, percentage body fat, total fat mass and body weight, as well as increasing age [114-116, 336]. Its release from adipocytes is dependent upon the volume of lipid within the cell, such that lipid-engorged adipocytes secrete significantly more leptin than smaller adipocytes [34]. This corresponds to data showing that more leptin is expressed and released from the larger subcutaneous adipocytes than from visceral depots [300, 337]. It is important to distinguish between the observation that IL-6-treated adipocytes were larger and contained more lipid but were not more numerous. The number of differentiating adipocytes in each sample, IL-6 treated and untreated, was approximately the same. Therefore, the increase in leptin secretion observed as a consequence of incubating IL-6 was related directly to the increased cell volumes in these cultures.

To determine a mechanism for the effect of IL-6 on adipogenesis, two rt-PCR techniques were used. Initially, end-point rt-PCR, a non-quantitative measure of expression, which amplifies a message a fixed number of times, was used. This provided some interesting results, and it was decided to confirm these with a more accurate and semi-quantitative method, real-time rt-PCR. Immediately noticeable was the fact that the results obtained by the latter method did not correlate with the original end-point data. End-point rt-PCR has a number of fallibilities that might affect the results: (i) it is not quantitative, therefore relying on a subjective observation of an agarose gel. This can be minimised by using computer-

based colour-density analysis of the image, but this method still requires a clear gel with clean bands; (ii) it is difficult to be certain that reactions have run correctly, in spite of the fact that analyses of the message are made prior to running samples to ensure that the number of cycles is within the linear phase of expansion. The difference in band intensity between one cycle and the next is often minimal, and the difference between obtaining no visible message and a plateaued intensity can be difficult to assess; and (iii) although reactions are repeated a number of times, only trends can be inferred. Data cannot be effectively pooled and compared to provide intra-sample variation and, again, results can depend on the success of the gel. Therefore, because these problems are not encountered when using real-time rt-PCR, the semi-quantitative data (which does not rely on gels or visual determination, or an arbitrary cycle number, and that provides clear mathematically serviceable results) are likely to be more reliable.

Therefore, using data obtained from real-time rt-PCR, it appears that IL-6 treatment does not increase the secretion of leptin by altering the expression of its mRNA. Although increasing expression would seem to be the most obvious method of increasing release, it is not the only possibility. As described in Chapter 3, leptin is stored in intracellular pools prior to secretion [287], and IL-6 might be directly affecting the exocytosis of these. This possibility is excluded, however, by the fact that shorter-term (48 h) incubation of mature adipocytes with IL-6 does not directly promote an increased secretion of leptin. It might be that leptin release is increased as a consequence of the effects of chronic exposure to IL-6 on adipogenesis. Continuous exposure to IL-6 had the effect of increasing the lipid content of the adipocyte (an effect not noticeable following 48 h exposure in mature adipocytes), and it could be through this that secretion is increased. A mechanism for this must be established, but it is possible to speculate that an increase in the post-transcriptional production of leptin occurs (or a reduction of proteasome-based degradation), or that leptin is stored in such excess in the intracellular pools that, although the increase in release is significant, it is not

sufficient to deplete these pools. The latter possibility warrants an investigation of the dynamics and kinetics of leptin secretion, especially considering that TNFα, at least in acute treatments, can upregulate leptin release without affecting expression [166]. The former depends upon the activity of leptin-degrading systems in the adipocyte, which are poorly reported (although leptin is degraded by this system [338], as are other components in the adipocyte [339-341]).

Although there was no significant effect on the expression of *ob*, other genes were affected by incubation with IL-6. Notably, the chronic exposure of differentiating adipocytes to IL-6 inhibits the expression of the potent adipogenic transcription factor PPARγ. A similar conclusion has previously been reached concerning the effects of adipokines and insulin resistance, in a study suggesting that IL-6, in addition to other cytokines, decreased the expression of PPARγ in adipocytes [266]. This discovery seems to contradict the observation that the morphology of IL-6 treated adipocytes is more lipid engorged, and that adipogenesis, at least in terms of lipid accumulation and leptin release, is driven primarily by PPARγ [59, 342, 343]. IL-6 is not completely inhibiting PPARγ expression, however, and C/EBPα, the second main protagonist of adipocyte differentiation, is unaffected. Therefore adipogenesis will still occur, and whereas the reduction in PPARγ has not negatively affected differentiation, it might have had a secondary effect on the expression of other adipocyte genes. PPARγ affects the expression of other genes; microarray and other methods of detection have revealed a massive list of targets for PPARγ [344-347]. Obviously these include a number of genes required for adipogenesis and the function of the mature adipocyte. Among those that are upregulated include: C/EBPα [54, 61]; LPL [348]; fatty acid binding protein-4 [105]; adipsin [28, 349, 350]; IR and IRS-1 [351]; aP2 [342]; STATS 1, 5A and 5B [352]; the glucose transporters GLUT1 [353] and GLUT4 [28]; aquaporin adipose [354]; and ApoA I and II [29]. A number of other adipocyte and metabolic genes are downregulated by PPARγ, including *ob* [53, 355, 356], ApoCIII [348, 357]

and resistin [358] and, in the case of adiponectin, there is still confusion as to whether it is up- [194, 359, 360] or downregulated [358] by PPARγ. Two of these gene products are of particular interest, leptin and adipsin.

Adipsin is an adipocyte marker, homologous to human complement factor D, that is expressed in adipocytes but not preadipocytes [72, 361]. The data presented here show that adipsin mRNA is significantly greater in adipocytes compared with preadipocytes from as early as three-days post-induction of differentiation; however, in the mature adipocyte its expression is reduced following exposure to IL-6. This might be as a consequence of the IL-6 reduction of PPARγ expression, which in turn results in a downregulation of adipsin expression. That this does not happen with *ob* expression is more of a quandary, as less PPARγ would be expected to equate with more leptin. However, although an increase in PPARγ has been demonstrated to decrease leptin secretion and expression [355, 356], the control of *ob* is also regulated by C/EBPα [53, 117, 362], which is the only predictor of leptin mRNA *in vivo* [363], and thus might preserve leptin gene expression [364]. This suggests that *ob* expression in mature adipocytes is more explicitly controlled by C/EBPα, which is unaffected by the presence of IL-6, than it is by PPARγ.

In addition to the use of dexamethasone, IBMX and insulin, a number of other methods are frequently used to differentiate 3T3-F442A cells. The most commonly cited method (after the adipogenic cocktail of dexamethasone, IBMX and insulin) is indomethacin and insulin, with T_3 and insulin also noteworthy. The protocols that are used vary in the mechanisms used to promote adipogenesis. In each case, insulin is used to enhance glucose uptake, and therefore lipid deposition. Insulin also has an effect on C/EBPα, with an upregulation of the expression of C/EBPα in the presence of insulin [364] (although the opposite effect has also been described [365]), but this is not the primary reason that insulin is included in the adipogenic cocktail. Insulin also increases the expression of glycerol-3-phosphate dehydrogenase [366, 367], *ob* [303,

364, 368] and HSL [369], and decreases the expression of adipsin [366] and resistin [178]. T_3 is used as a potentiator of insulin action, increasing glucose uptake [370] and lipogenic factors for lipid deposition [283, 371]. In contrast to the other methods described, the T_3 and insulin protocol does not contain a factor specifically included to enhance the expression of differentiation-related genes, instead relying on the spontaneous conversion of preadipocytes and the possible effect of insulin on C/EBPα. Indomethacin, a cyclooxygenase inhibitor and non-steroidal anti-inflammatory drug, is a potent activator of the adipogenic transcription factor PPARγ [87], and subsequently enhances fatty acid and triglyceride synthesis [86]. Dexamethasone enhances the expression of the early adipogenic transcription factor C/EBPδ [73], causes growth arrest [75] and downregulates the expression of the anti-adipogenic factor *pref-1* [78], as well as potentiating the effects of cAMP [77], the target of IBMX. IBMX itself is used as a non-specific phosphodiesterase inhibitor, to increase the activity of glycerophosphate dehydrogenase and, therefore, lipid deposition [80, 372]. The aim of all the protocols described is to increase the expression of adipogenic genes and to enhance lipid deposition.

Of the protocols investigated here, which also included one method eschewing dexamethasone in favour of a double dosage of IBMX, the success in differentiating 3T3-F442A cells differed considerably. It is clear that IL-6 alone is not sufficient to induce adipogenesis, as demonstrated by its failure to induce the expression of either PPARγ or C/EBPα. In this study the use of T_3 and insulin was relatively ineffective at differentiating 3T3-F442As, in terms of both adipose conversion and leptin release. This is probably owing to the lack of a dedicated agent (as with cells treated with only IL-6) designed to induce C/EBPα or PPARγ expression. Similarly, the IBMX method in the absence of dexamethasone was less effective than that using dexamethasone, probably for the same reasons. By contrast, differentiation using indomethacin was more effective, in terms of both the recruitment of adipocytes and leptin release, than the 'baseline' standard

of dexamethasone and IBMX. This is probably a result of its direct interaction with the potent adipogenic transcription factor PPARγ, increasing the recruitment of the preadipocytes and enabling more cells to differentiate quickly and efficiently.

The results reported here are noticeably different to the only published comparison of differentiation-dependent leptin release from cell lines [284], which observed virtually the opposite of what was seen here. In the previous study, T_3-treated cells released the greatest levels of leptin, and indomethacin-treated cells secreted the least. Additionally, greater leptin release was observed from 3T3-L1 adipocytes compared with 3T3-F442A cells. This is unusual, because a number of studies have investigated leptin expression or release from 3T3-F442A adipocytes differentiated using dexamethasone, IBMX and insulin, and have recorded appreciable levels [368]. It does appear that the conditions of differentiation significantly affect leptin release [373]. Previous studies have consistently identified insulin as a promoter of leptin secretion and expression [281, 303, 374]. This appears to be contradicted in the Slieker *et al.* study [284], in which extremely low levels of insulin (as used in the T_3 and insulin protocol) promote increased *ob* expression when compared with the methods using a higher concentration of insulin. Furthermore, high concentrations of glucose reduce the expression of *ob* and inhibit the action of insulin on adipocytes [375]. The Slieker study involved a glucose concentration of 25 mM. This is equivalent to 4500 mg/L, considerable greater than the 2500 mg/L concentration that was used in this study: a concentration that is described as 'high' by its manufacturer. Unfortunately, Slieker *et al.* [284] provided no data concerning the differentiation state of the adipocytes in their study. If the indomethacin- or dexamethasone-containing protocols resulted in adipocytes that were less lipid engorged, as would be expected if insulin function was impaired, then the lower expression of leptin would not be surprising [34]. The inclusion of T_3, an insulin potentiator, in the more successful protocol might negate the inhibitory effects of excess glucose on insulin. This would enable greater lipid deposition and, consequently, greater *ob*

expression. In the absence of data concerning the adipocyte morphology, very little information concerning a possible mechanism can be gleaned. In the data presented here, leptin release broadly corresponds to the size of the adipocytes (assessed by microscopy), in agreement with previously published reports.

The effect of IL-6 on the various differentiating methodologies is curious. The pattern that was established with the dexamethasone, IBMX and insulin protocol, in which leptin release was increased when the cells are co-incubated with IL-6, was preserved for the dexamethasone-free IBMX treatment. There was no effect of IL-6 on leptin secretion from the cells that were differentiated using T_3 and insulin, and the presence of IL-6 resulted in a decrease in leptin release from cells that were treated with indomethacin and insulin. The T_3 observation could be attributed to the low levels of leptin recorded. The effect of IL-6 on leptin release from adipocytes that were differentiated using dexamethasone, IBMX and insulin was more prominent as differentiation continued: the adipocytes were larger (i.e. more lipid engorged) and released considerably greater levels of leptin. Had the time-course of the T_3 protocol been extended and the volume of the adipocytes increased, with a corresponding increase in leptin release, a difference might have emerged. The reduction in leptin secretion that was observed when IL-6 was present during differentiation with indomethacin and insulin might be as a result of the IL-6 effect on PPARγ, in which PPARγ expression was marginally inhibited by IL-6. This would imply that IL-6 is interfering with the indomethacin-mediated upregulation of PPARγ, with a minor reduction in PPARγ levels affecting adipose conversion rates. Confirmation by way of measuring mRNA levels is required to confirm this.

Therefore, adipocytes differentiated using dexamethasone, IBMX and insulin that were also treated with 10 ng/ml IL-6 exhibit increased lipid deposition and leptin secretion without directly affecting adipogenesis. IL-6 had no explicit effect on the expression of *ob*, the gene that encodes leptin, but reduced the mRNA levels of the transcription factor PPARγ, possibly affecting adipsin expression. IL-6

also appears to hinder indomethacin-induced differentiation, and although the mechanism of this is unknown, the IL-6 effect on PPARγ might be involved. There appears to be no direct genetic mechanism for the observed increase in leptin release, although the data reported in Chapter 5 pose some more possibilities.

Cell volume is a significant risk factor for pathologies associated with obesity. One such pathology, insulin resistance, is caused (at least in part) by adipokines, and is related to an increase in adipocyte volume rather than number [31, 32]. Significantly, IL-6 has been shown here to increase the lipid content of differentiating adipocytes. Similarly, the work of Mattacks *et al.* [376] suggests that adipocytes in close proximity to lymph nodes are susceptible to HIV or lipopolysaccharide-associated hypertrophy. It is possible, especially when considering the data presented here, that IL-6 might be responsible (at least partially) for this adipocyte hypertrophy, because levels of IL-6 are significantly increased (from non-adipocyte sources) following LPS challenge or in HIV.

Adipocyte hypertrophy is relevant because insulin resistance is more closely related to visceral adipose tissue than subcutaneous [30]. As described in Chapter 3, visceral adipose tissue from obese individuals releases more IL-6 than does subcutaneous. Therefore, although it appears unlikely that IL-6 has a direct mechanistic effect on insulin resistance (Chapter 5), the strong correlation between the two might be as a result of the feedback relationship in visceral tissue.

Chapter 5 – Glucose Uptake and Lipolysis

Introduction

To fulfil its role as an energy store for the body, adipose tissue must be able to satisfy three criteria: it must have the ability to take up substrates in times of excess energy input, the ability to release substrates for peripheral tissues in times of fasting and have an effective method of storing energy to implement both uptake and release. This third capability is achieved by the use of triglyceride (also known as triacylglycerol) as the predominant long-term storage molecule in the body. Triglycerides consist of three long-chain fatty acid moieties bound to a glycerol backbone. They are non-polar, and are thus stored in an anhydrous form (glycogen, the storage form of glucose is stored in a hydrated form, requiring up to twice its dry weight in water), meaning that the energy storage to weight ratio is very high. Additionally, the lower oxidation state of carbon in fats when compared with carbohydrates results in greater energy release upon oxidation than from carbohydrates [377]. When the organism is in a fed state (i.e. a positive energy state), triglycerides are formed within the adipocyte, from fatty acids or glucose taken up from the bloodstream. When the organism is in the fasted state (a negative energy state), the triglycerides are hydrolysed into their constituent fatty acids and glycerol and are released from the adipocyte into the circulation.

Lipids are stored within the adipocyte in a specialised lipid vacuole, which is characterised by its high triglyceride content, with the remainder of the droplet consisting of protein and phospholipids [378]. Approximately 5% of the lipid-droplet mass is phospholipid, which forms the surface monolayer of the vacuole. A further 5% of the mass is protein, predominantly perilipins and adipose differentiation-related protein (ADRP), which share amino-terminus homology, and their presence depends upon the stage of differentiation [379]. Perilipins are found on the periphery of lipid vacuoles in mature adipocytes [380, 381] and appear to be involved in the packaging of lipids [382]. Perilipin levels correspond to those of triglycerides [383] and the expression of perilipins might be under the control of lipids in a similar mechanism to ADRP

[384]. In addition to the packaging role, perilipins are involved in HSL-related lipolysis. The remainder of the lipid content is triglyceride, with small quantities of free fatty acids and β-carotene also present [385].

Lipogenesis and glucose uptake

Lipogenesis, which is the formation of triglycerides within the adipocyte, is controlled by a number of enzymatic processes. Very little triglyceride is taken up by the adipocyte directly. Instead, triglyceride from the bloodstream is digested by the enzyme LPL into fatty acids, which can diffuse into the cell. A second enzyme, fatty acyl-CoA synthase, is then responsible for converting these fatty acids to triacylglycerol, to prevent diffusion from the cell and for storage. This is the preferential form of triglyceride synthesis in the cell and the mechanism by which most is formed [377].

Alternatively, triglycerides can be formed *de novo* from acetyl-CoA and malonyl-CoA precursors. For this synthesis, glucose is the base substrate for acetyl-CoA, as well as the glycerol-3-phosphate backbone. Glucose is metabolised via the glycolysis pathway, and is converted to acetyl-CoA in the Krebs cycle or to glycerol-3-phosphate by glyceraldehyde-3-phosphate during glycolysis. Subsequently, the multifunctional enzyme fatty acid synthase adds malonyl-CoA (itself formed from acetyl-CoA by acetyl-CoA carboxylase) sequentially to an acetyl-CoA substrate, to form palmitic acid, which is the predominant fatty acid used for storage in triglycerides (Figure 5.1) [386]. Other fatty acids are produced by the elongation or desaturation of this palmitic acid precursor [387]. These fatty acids are then transferred to a molecule of glycerol-3-phosphate to form monoacylglycerols, with the addition of a second and third fatty acid moiety producing a diacylglycerol and triacylglycerol respectively. These reactions are controlled by various acyl-transferases [388].

However, unlike fatty acids, glucose is unable to passively diffuse through the cell membrane. Instead, glucose uptake is mediated by a family of membrane-bound channels known as the glucose transporters

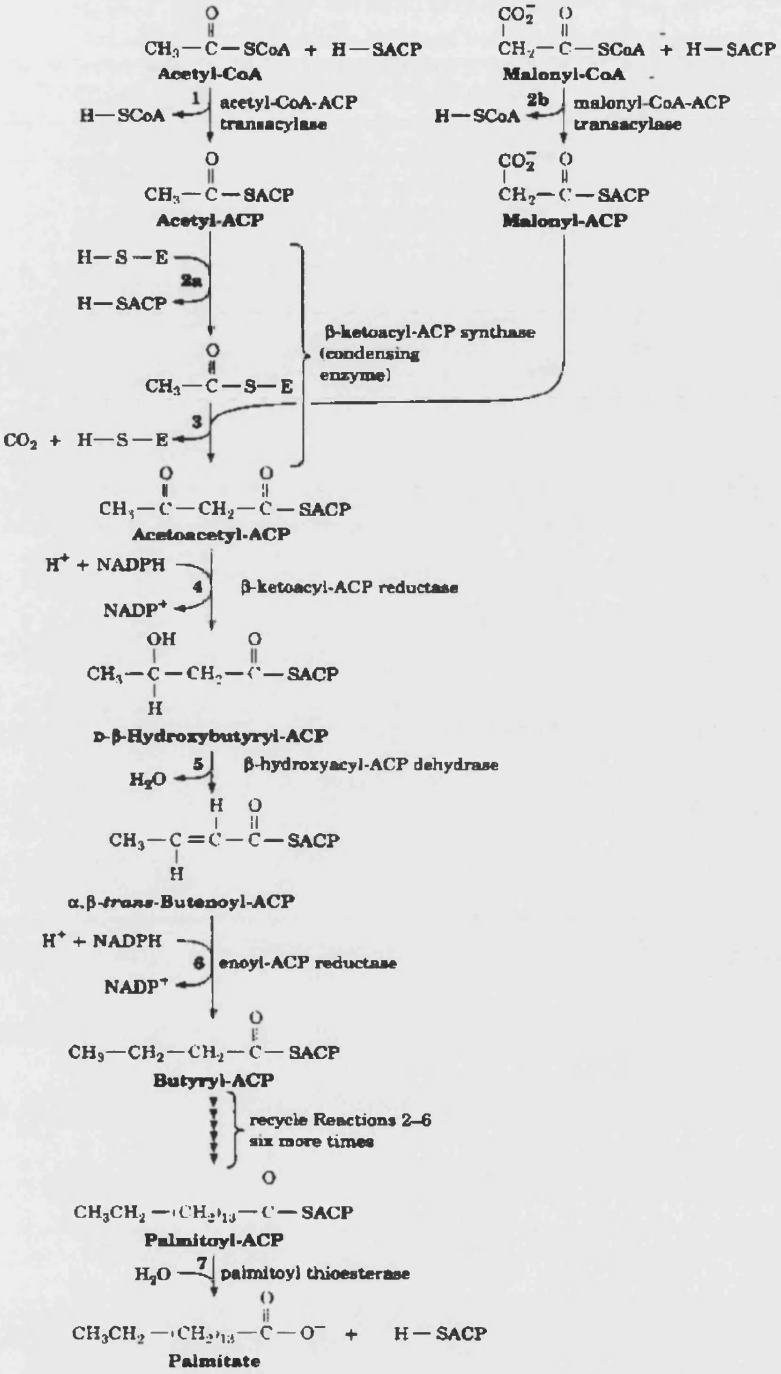

Figure 5.1 – The formation of palmitate from acetyl CoA and malonyl CoA

From Voet and Voet [377].

(GLUTs). Six members of the GLUT family have been described (GLUTs 1–5 and GLUT 7) [389], all of which are transmembrane proteins with a membrane-spanning region providing an aqueous pore for glucose transport [390]. In adipocytes, the predominant transporters are GLUT1 and GLUT4, which are insulin independent and insulin dependent, respectively. In an unstimulated adipocyte, the majority (95%) of GLUT4 is located in intracellular pools, as is 30–40% of GLUT1, where they are non-functional. Their redistribution to the plasma membrane is necessary for function [391].

The best-studied mechanism for the stimulation of glucose uptake is that which occurs in response to insulin. Insulin is produced by the β-cells of the pancreas in response to the dietary intake of carbohydrate. Insulin binds to IRs on the adipocyte membrane and this complex interacts with IRS-1. This results in the tyrosyl phosphorylation of IRS-1 and the subsequent phosphorylation and activation of intracellular kinases, such as phosphatidylinositol-3-kinase [392, 393]. The relocation of GLUT4 to the plasma membrane enables glucose translocation, providing the substrate for lipogenesis [394-396]. However, chronic exposure to insulin downregulates the expression of GLUT4 but upregulates GLUT1 transcription, causing an increase in constitutive glucose uptake and reducing the capacity for insulin-stimulated uptake.

Interest in the effects of adipokines on glucose uptake and (specifically) insulin action developed from the discovery that adipocyte-derived TNFα affects insulin action. Insulin-stimulated glucose uptake is severely restricted in the condition of insulin resistance, which is observed in many cases of obesity and type-2 diabetes. Even prior to the identification and naming of the molecule, TNFα was implicated in the pathogenesis of insulin resistance [397]. Correlations between adipocyte expression of TNFα and insulin resistance were subsequently reported [102, 161]. TNFα directly inhibits insulin signalling in adipocytes by a variety of mechanisms, specifically by reducing the protein or mRNA levels of GLUT4 [168, 171, 398, 399] or by inhibiting the tyrosine phosphorylation of IR and IRS-1 [167, 296, 400-402]. Subsequently, other

adipokines have been reported to affect insulin action. Leptin, under certain conditions (the excess levels of leptin that are observed in obesity appear to result in leptin resistance, thus severely reducing its functions [133]), enhances insulin action and reverses insulin resistance [403-406]. More recently, adiponectin has been shown to alleviate insulin resistance and to increase glucose uptake [188, 407-409]. The effects of IL-6 are currently disputed (see discussion).

Lipolysis

During times of fasting, tissues, such as cardiac and skeletal muscle and the liver, require substrate for energy production. Adipocytes can respond to these requirements by hydrolysing triglyceride into its component glycerol and fatty acids. Fatty acids are then free to diffuse out of the cell to be transported to where required. The rate-limiting step of lipolysis is controlled by HSL, a multi-enzyme complex that hydrolyses individual fatty acid moieties from glycerol. As its name suggests, HSL is influenced by endocrine signals, increasing or reducing lipolysis as necessary.

HSL appears to be controlled by the phosphorylation and dephosphorylation of serine residues on the enzyme [410]. Site-directed mutagenesis has identified a number of serine residues that, when phosphorylated, will activate or inactivate the protein [411]. A second mechanism that controls HSL is the intracellular location of the enzyme. Following stimulation with a lipolytic signal and subsequent phosphorylation, HSL is translocated from the cytoplasm to the lipid droplet [412]. Further control is imparted by perilipin, the lipid vacuole protein, which is associated with HSL following its translocation. A number of mechanisms are related to the function of perilipin in lipolysis. Concurrent phosphorylation of perilipin A with HSL appears to enable the hydrolysis of triglycerides [413], but perilipins also have a role in preventing lipolysis [414], possibly by preventing HSL from coming into contact with stored lipids [285]. The inhibition of lipolysis conferred by perilipin is so significant that animals lacking perilipin are immune to diet-induced obesity [415]. Therefore, the control of the lipolytic function of

HSL occurs at many levels and many of the mechanisms remain unresolved.

The stimulation of lipolysis is caused by many effectors, including catecholamines, prostaglandins, TNFα, IL-1, IFNα, IFNβ and IFNγ. Catecholamines act through β-adrenoceptors, resulting in the activation of adenylate cyclase, thus increasing intracellular cAMP and activating cAMP-dependent protein kinase (PKA). PKA phosphorylates the serine residues on HSL that are required to activate the enzyme. The cytokines appear to stimulate lipolysis by inducing prostaglandin synthesis, because their effect is inhibited by the prostaglandin inhibitor indomethacin [270]. Conversely, glucose can inhibit the action of HSL by decreasing the expression of the enzyme [416]. Insulin reduces PKA activity and, therefore, HSL phosphorylation, although prolonged exposure to both insulin and glucose increases HSL expression, which might explain the increased lipolysis that is observed in diabetic patients with hyperglycaemia and hyperinsulinaemia [369]. As with glucose uptake, IL-6 has been reported to have a variety of effects on lipolysis.

As with the adipogenesis methods, the hypothesis was that IL-6 would act similarly to TNFα; that is, increase lipolysis and inhibit glucose uptake (although one report, Stouthard *et al.* [263], appeared to indicate the opposite: this was to be confirmed or rejected by this study).

The aims of this chapter, therefore, were to investigate:
(i) The effect of IL-6 on basal and insulin-stimulated glucose uptake.
(ii) The effect of IL-6 on basal and adrenoceptor-stimulated lipolysis.
(iii) The possible mechanisms of these actions.

Methods

Glucose Uptake

Differentiated 3T3-L1 and 3T3-F442A adipocytes were used to assess glucose uptake. Cells that were used for ^{3}H-DOG uptake measurement were grown and differentiated in 6-well plates. Insulin was withdrawn from the DMEM/CCS (to re-sensitise the cells to insulin) for 24 h or 48 h, followed by treatment in the absence or presence of 10 ng/ml IL-6 in 1.5 ml/well Cell-gro for a further 24 h or 48 h. The media was refreshed every 24 h, where necessary. Following incubation, cells were washed and treated for 30 minutes with or without 1 μg/ml insulin in Cell-gro (volumes as before). For the final ten minutes of the uptake-measurement experiment, 1 μl/ml media ^{3}H-DOG was added to each well. After the 30-minute incubation, the media was removed and discarded (Figure 5.2). Adipocytes were washed with ice-cold KRP buffer and digested using 0.1% SDS. 800 μl of the cell homogenate was removed, added to 8 ml scintillation fluid and counted.

Data for glucose uptake are expressed as mean fold-increase (standard deviation) in uptake of ^{3}H-DOG compared with basal (no IL-6), and significance was assessed by Mann-Whitney U test, where two-tailed *p* values below 0.05 were considered significant. Experiments were repeated a minimum of three times.

Insulin receptor signalling

Because 3T3-L1 adipocytes responded better to insulin than did 3T3-F442A cells, western-blot analysis for insulin signalling proteins, and the phosphorylation state of these proteins using immunoprecipitation, was carried out only with 3T3-L1 cells. Adipocytes for insulin-receptor signalling were grown in 75-cm^{2} tissue culture flasks. Using a method similar to that for glucose uptake, insulin was withdrawn for the DMEM/CCS culture media for 48 h, followed by treatment in the absence

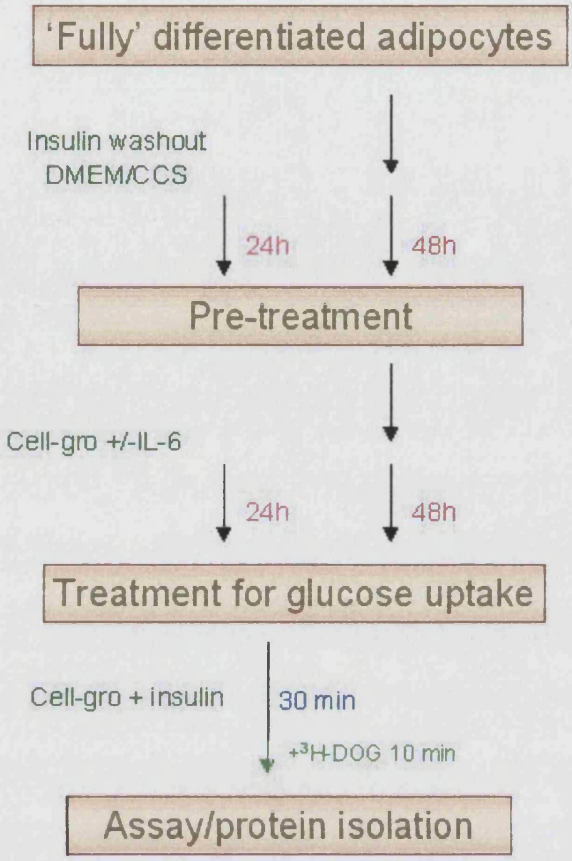

Figure 5.2 – Time line of glucose uptake experiments.

Differentiated adipocytes were treated for 24 h or 48 h in DMEM/CCS to re-sensitise the cells to insulin. Cells were then pre-treated for an equal length of time in Cell-gro, in the absence or presence of 10 ng/ml IL-6. Glucose uptake treatment was 30 minutes in the presence of insulin, with ^{3}H-DOG added for the final 10 minutes. Cells were then solubilised for assay and protein isolation.

or presence of 10 ng/ml IL-6 in 10 ml Cell-gro. After 48 h, the cells were treated for glucose uptake as described above, the culture media was aspirated and the flasks flash-frozen in liquid nitrogen Flasks were stored at -80°C prior to analysis.

Western blotting of IRβ and IRS-1, and IRS-1 phosphorylation, as well as subcellular localization of GLUT1 and GLUT4, was carried out as described in Chapter 2. Exposure times for each of the products were: IRβ, IRS-1 and IRS-1 phosphorylation, 1 minute; GLUT1, 15 seconds; GLUT4, 45 seconds.

Lipolysis

Lipolysis was measured as glycerol concentration released into the cell-culture supernatant of 3T3-F442A adipocytes. The experiments were also carried out on 3T3-L1 cells, but this cell line did not release sufficient levels of glycerol for assay. Adipocytes were grown, differentiated and treated in 6-well plates. The majority of the experiments were carried out on adipocytes differentiated using dexamethasone, IBMX and insulin. As a comparison, a subset of experiments was carried out on cells differentiated using IBMX and insulin, or indomethacin and insulin. Prior to the lipolysis experiment, cells were pre-treated for 8 h, 12 h, 24 h, 48 h and 72 h in 1.5 ml/well Cell-gro, in the absence or presence of 10 ng/ml IL-6. Cells were then treated for 6 h with 1.5 ml/well Cell-gro containing 1 μM dobutamine (a β1-adrenoceptor agonist), 1 μM clenbuterol (a β2-adrenoceptor agonist), 1 μM CL316243 (a β3-adrenoceptor agonist), 1 μM noradrenaline (a non-specific α/β agonist), 2.5 μM dbcAMP (cAMP analogue) or with no additives, in the absence or presence of 10 ng/ml IL-6. Following 6 h incubation, the media was removed and assayed for glycerol release as described in Chapter 2 (Figure 5.3). The adipocytes were homogenised in TRI-reagent for RNA isolation (see Chapter 2). RNA was used for northern analysis of the samples, using probes for β2-adrenoceptors and β3-adrenoceptors, and to synthesise cDNA for rt-PCR analyses of both β2- and β3-adrenoceptor message.

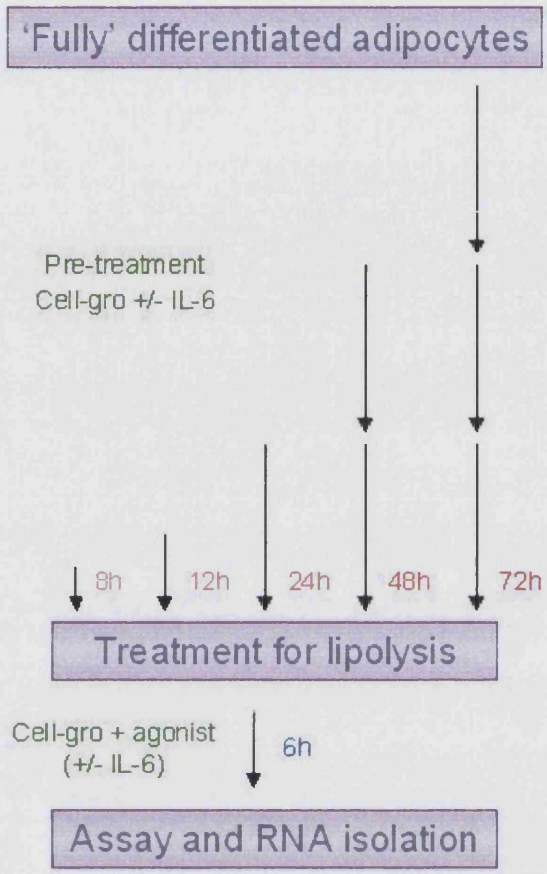

Figure 5.3 – Time line of lipolysis experiments.

Differentiated adipocytes were pre-treated for 8–72 h in Cell-gro, in the absence or presence of 10 ng/ml IL-6. Cells were then subjected to a 6 h treatment with adrenergic agonists or without stimulant (in the absence or presence of IL-6, as in the pre-treatment). Supernatants were assayed for glycerol release and cells used for RNA isolation.

Data are presented as percentage increase (interquartile range) in glycerol levels compared with basal (no IL-6) for each pre-incubation time period, unless otherwise stated (*n* values refer to the number of individual wells from which measurements were obtained). Significance was assessed by Mann-Whitney U test, and two-tailed *p* values below 0.05 were considered significant. Real-time rt-PCR data are expressed as relative difference in expression (interquartile range) compared with basal (no IL-6), using the method described by Livak and Schmittgen [295]. All experiments were repeated a minimum of three times.

Results

The effect of IL-6 on glucose uptake

Changes in insulin-stimulated glucose uptake in 3T3-L1 adipocytes after 48 h of insulin withdrawal (that is, 24 h in Cell-gro followed by 24 h in the absence or presence of IL-6) were variable. However, a total of 96 h of insulin withdrawal rendered the adipocytes insulin responsive. Insulin treatment resulted in a mean (standard deviation) 4.1 (1.3)-fold increase in glucose uptake compared with basal ($p< 0.031$; Figure 5.4). IL-6 pre-treatment increased glucose uptake 3.1 (2.4) fold ($p= 0.05$) and insulin treatment following incubation with IL-6 caused a 14.7 (3.4)-fold increase in glucose uptake compared with basal ($p< 0.001$).

The F442A adipocytes were considerably less responsive to insulin. Following 96 h insulin withdrawal, glucose uptake compared with basal was enhanced 1.9 (0.1) fold in response to insulin ($p< 0.001$), 1.5 (0.2) fold with IL-6 treatment ($p< 0.001$) and 3.0 (1.0) fold in response to insulin after IL-6 pre-treatment ($p< 0.001$).

Notably, the basal uptake of glucose was similar in both cell lines, at both time points [3T3-L1 48 h insulin withdrawal, median (interquartile range) 24371 (19001–29231) counts per minute (cpm); 3T3-L1 96 h insulin withdrawal, 25480 (15215–43320) cpm; 3T3-F442A 48 h insulin withdrawal, 28294 (21953–30164) cpm; 3T3-F442A 96 h insulin withdrawal, 31751 (29502–36020) cpm. All $p=$ ns, with the exception of 3T3-L1 48 h vs. 3T3-F442A 96 h, $p= 0.01$].

The effect of IL-6 on proteins related to glucose uptake

To investigate possible mechanisms for the effect of IL-6 on glucose uptake, changes in the protein levels of components of the insulin-signalling pathway were measured. Figure 5.5 shows the comparative protein levels of IRβ, IRS-1 protein and IRS-1 phosphorylation following each of the treatments that were used to induce glucose uptake. Treatment with insulin appeared to increase the levels of IRS-1 compared

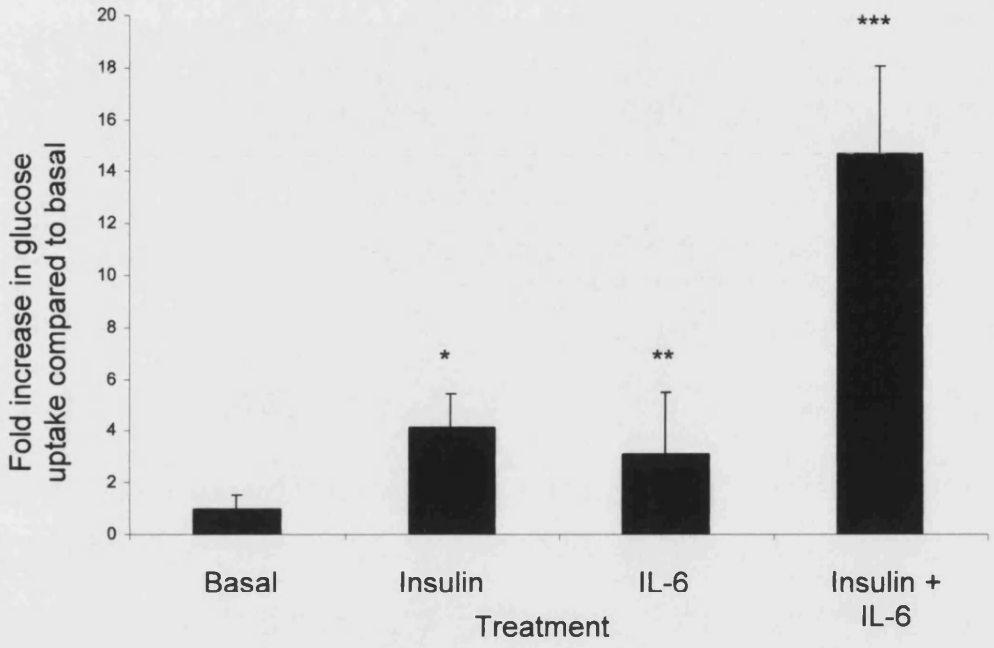

Figure 5.4 – The effect of IL-6 on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes.

The effect of 48 h pre-incubation (following a 48 h insulin washout period) with 10 ng/ml IL-6 on basal and insulin stimulated glucose uptake in 3T3-L1 adipocytes, shown as mean fold increase compared with basal (no IL-6), + SD (n= 9 for each sample).

*p= 0.031; **p= 0.05; ***p< 0.001 (compared with basal).

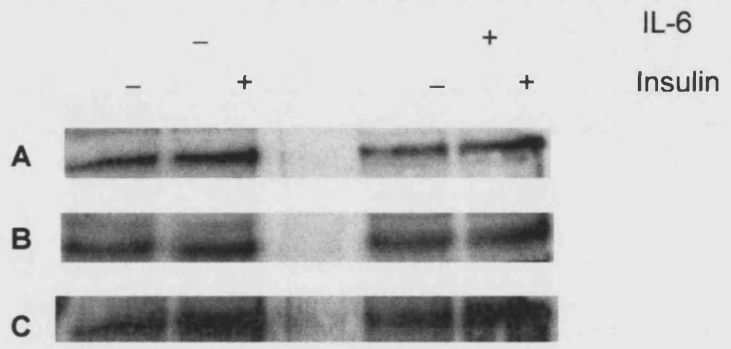

Figure 5.5 – The effect of IL-6 on insulin signalling during glucose uptake.

The effect of 48 h (following a 48 h insulin washout period) pre-incubation with 10 ng/ml IL-6 on IRS-1 **(A)** and insulin receptor-β **(B)** protein, and IRS-1 phosphorylation **(C)**, during basal (insulin –) and insulin-stimulated (insulin +) glucose uptake.

with basal, and clearly upregulated its phosphorylation without affecting the levels of IRβ. IL-6 pre-treatment had no effect on any of these proteins, whether adipocytes were treated with insulin or not. Therefore another mechanism must be involved.

Glucose transport into adipocytes is facilitated by the two glucose transporters GLUT1 and GLUT4, which are only functional when located at the plasma membrane. Incubation with IL-6 had no effect on the protein levels of either GLUT1 or GLUT4 in the adipocyte (Figure 5.6). Unfortunately, attempts to analyse GLUT translocation in response to insulin and/or IL-6 were not successful during the time available.

The effect of IL-6 on basal and stimulated lipolysis

A comparison of the basal (unstimulated) release of glycerol is shown in Figure 5.7. The peak release of glycerol was observed following 12–24 h pre-treatment with serum-free media. At both of these time points, significantly more glycerol was accumulated than after 8 h [167.9 (149.4–193.7) μg/ml; vs. 12 h, 275.4 (11.2; mean and standard deviation, owing to the lower sample number) μg/ml, $p= 0.02$; vs. 24 h, 276.1 (227.1–360.1) μg/ml, $p= 0.012$], 48 h [110.4 (99.6–183.2) μg/ml; vs. 12 h, $p= 0.007$; vs. 24 h, $p< 0.001$] or 72 h pre-incubation [22.9 (17.3–31.7) μg/ml; vs. 12 h, $p= 0.02$; vs. 24 h, $p= 0.001$]. There was no significant difference in glycerol accumulation between pre-treatment for either 12 h and 24 h or 8 h and 48 h. Levels of glycerol release following 72 h pre-incubation were barely above background, and also significantly below both 8 h ($p= 0.004$) and 48 h ($p= 0.001$) pre-incubation. This indicates that 48 h was the maximum pre-incubation period for viable lipolysis experiments carried out in these studies.

Unstimulated and stimulated lipolysis in the absence and presence of 10 ng/ml IL-6 following a 12 h serum starvation is shown in Figure 5.8. Treatment with any of the adrenoceptor agonists increased lipolysis (measured by glycerol secretion) compared with basal. Incubation with IL-6 during the pre-incubation and treatment had no effect on the

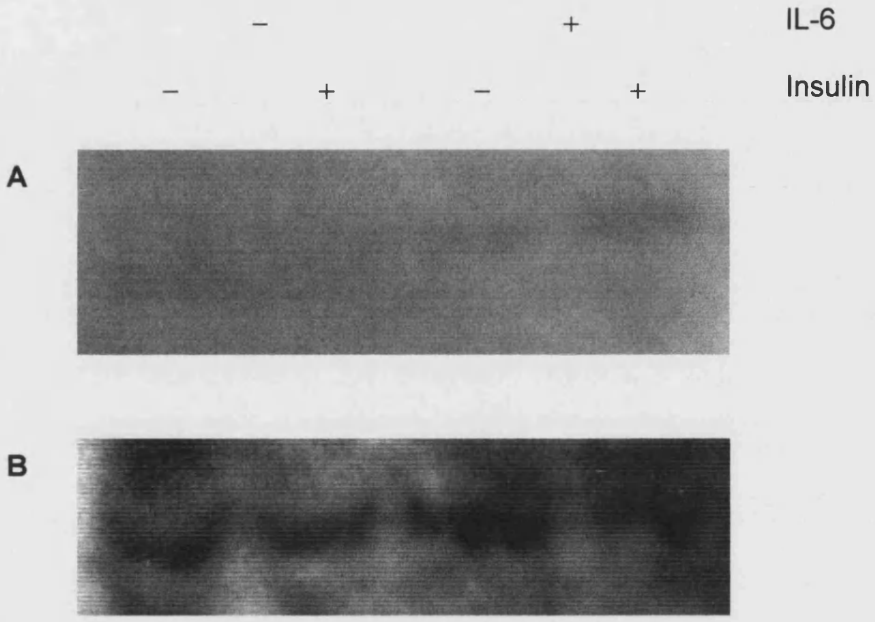

Figure 5.6 – The effect of IL-6 on glucose transporter levels during glucose uptake.

The effect of 48 h pre-incubation with 10 ng/ml IL-6 on GLUT1 **(A)** and GLUT4 **(B)** protein levels during basal (insulin –) and insulin-stimulated (insulin +) glucose uptake.

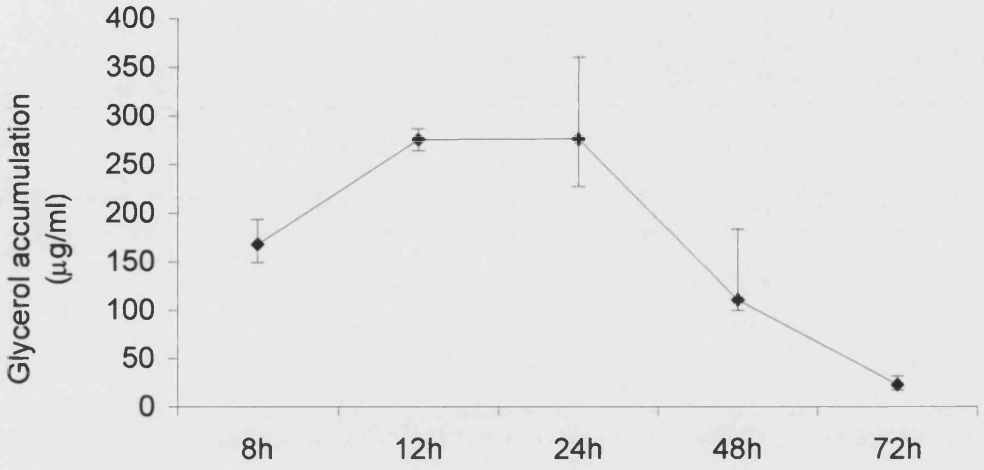

Figure 5.7 – Glycerol accumulation from unstimulated 3T3-F442A adipocytes.

The release of glycerol over 6 h from fully differentiated 3T3-F442A adipocytes, pre-treated in serum-free media for 8 h to 72 h. Data presented as median and interquartile range (12 h data presented as mean and standard deviation, owing to the lower sample number) of glycerol accumulated in the supernatant, without stimulation ($n \geq 6$ for each sample: 12 h, $n = 3$).

p values: see text.

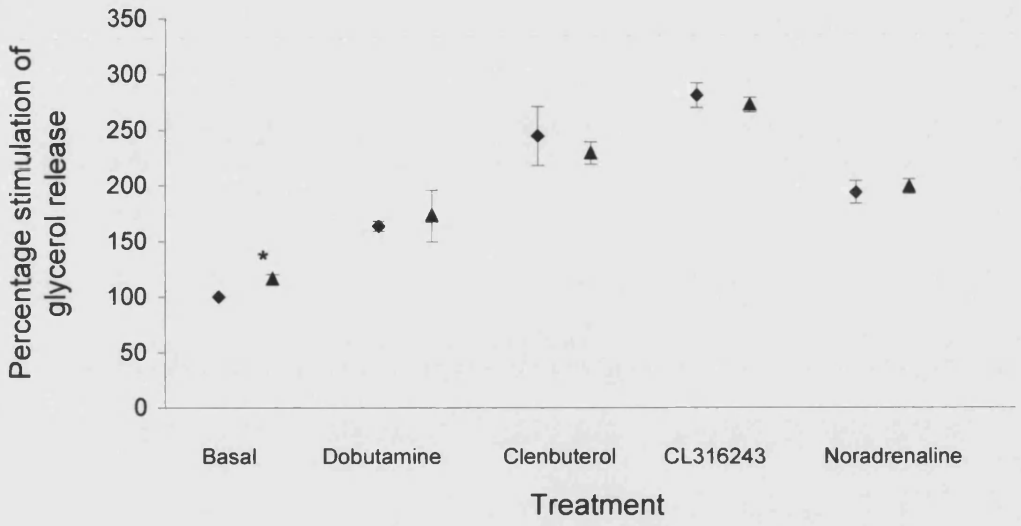

Figure 5.8 – Glycerol accumulation from 3T3-F442A adipocytes pre-treated for 12 h $^{+}$/- IL-6.

The release of glycerol from 3T3-F442A adipocytes pre-treated in serum-free media for 12 h in the absence (♦) or presence (▲) of 10 ng/ml IL-6, prior to 6 h incubation with a β1- (dobutamine), β2- (clenbuterol), β3- (CL316243) or a non-specific α/β-adrenoceptor agonist (noradrenaline), or no stimulant. Data presented as percentage stimulation (mean and standard deviation, owing to the low sample number) of glycerol accumulation compared with basal (no IL-6). Cells differentiated using dexamethasone, IBMX and insulin. *n*= 3 for each sample.

**p*= 0.05 compared with basal, no IL-6.

stimulated release of glycerol but significantly increased glycerol release in unstimulated samples [IL-6 treated 116.1% (4.1; mean and standard deviation) of basal, $p = 0.05$].

A pre-incubation of 24 h produced dramatically different results (Figure 5.9). In contrast to the results obtained following the 12 h pre-incubation, there was no significant effect of IL-6 on basal lipolysis [IL-6 treated release 105% (102.5–113.3) of basal, $p = 0.198$], whereas all stimulated glycerol release was inhibited following IL-6 treatment [IL-6 treated vs. untreated; dobutamine, 136.7% (124.7–153.9) vs. 173.6% (168.9–174.5), $p = 0.004$; clenbuterol, 211.4% (197.7–248.0) vs. 291.7% (279.1–320.8), $p < 0.001$; CL316243, 228.8% (195.1–286.6) vs. 339.6% (291.8–356.5), $p = 0.013$; and noradrenaline, 173.8% (159.4–211.8) vs. 225.1% (191.0–256.4), $p = 0.028$].

Following 48 h pre-incubation, only clenbuterol-stimulated lipolysis was significantly inhibited by IL-6 (IL-6 treated vs. untreated; 174.8% (159.2–186.3) vs. 251.4% (223.2–276.3), $p < 0.001$), with no other agonists affecting glycerol accumulation. Pre-incubation with IL-6 for 8 h had no effect on the lipolysis that was stimulated by any of the agonists, and the levels of glycerol release following 72 h pre-incubation were too low for meaningful analysis.

The effect of differentiation method on lipolysis

A similar series of experiments to those described above were carried out on 3T3-F442A adipocytes, which were differentiated using IBMX and insulin, or indomethacin and insulin. Pre-treatment in the presence or absence of IL-6 was for either 16 h or 24 h. Differentiation with IBMX and insulin resulted in adipocytes releasing significantly less glycerol than did cells that were differentiated with dexamethasone, IBMX and insulin (measured following 12 h or 24 h pre-incubation) following either 16 h or 24 h pre-incubation in serum-free media. Levels of unstimulated release after 16 h were barely above measurements of background glycerol secretion. After 24 h pre-incubation, significant responses (in the absence of IL-6) were only recorded in cells that were treated with clenbuterol

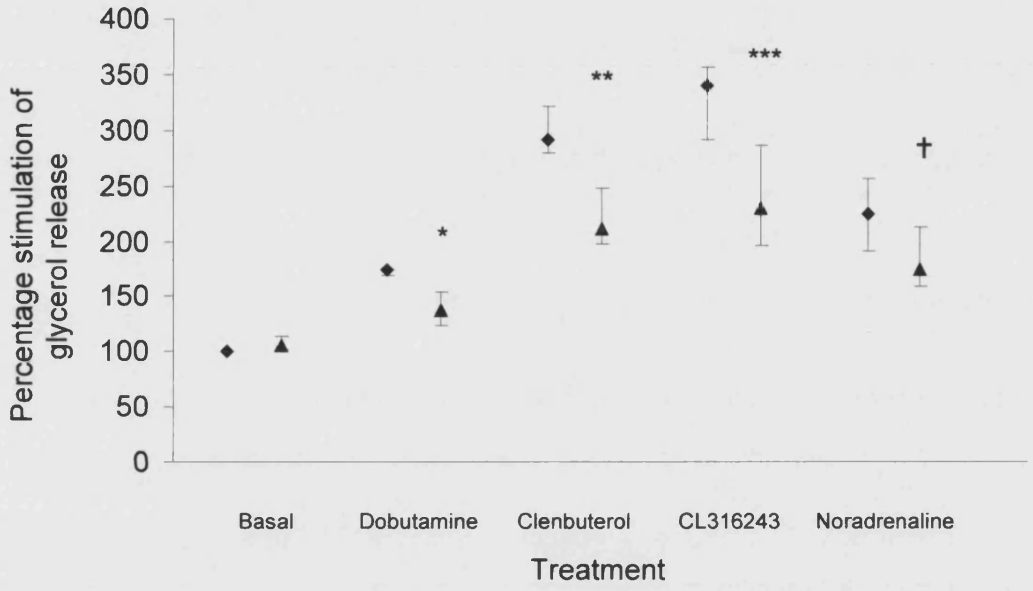

Figure 5.9 – Glycerol accumulation from 3T3-F442A adipocytes pre-treated for 24 h $^{+}$/- IL-6.

The release of glycerol from 3T3-F442A adipocytes pre-treated in serum-free media for 24 h in the absence (♦) or presence (▲) of 10 ng/ml IL-6, prior to 6 h incubation with a β1- (dobutamine), β2- (clenbuterol), β3- (CL316243) or a non-specific α/β-adrenoceptor agonist (noradrenaline), or no stimulant. Data presented as percentage stimulation (median and interquartile range) of glycerol accumulation compared with basal (no IL-6). Cells differentiated using dexamethasone, IBMX and insulin. *n*= 11 for each sample (*n*= 13 for basal no IL-6).

**p*= 0.004; ***p*< 0.001; ****p*= 0.013; †*p*= 0.028.

[287.1% (186.0–438.2) of basal, p= 0.043] and CL316243 [340% (245.5–444.7) of basal, p= 0.021], and there was no significant effect of IL-6.

Adipocytes differentiated using indomethacin and insulin were more responsive to lipolytic stimuli than those that were differentiated using IBMX and insulin. At both 16 h and 24 h, a significant response to each of the agonists was observed, and unstimulated release was greater than that of IBMX and insulin differentiated cells [glycerol release from indomethacin vs. IBMX treated cells, 24 h pre-treatment; 140.6 (136.8–176.3) vs. 44.3 (21.9–71.6) μg/ml, p= 0.021]. There was no significant difference in unstimulated glycerol release following 24 h pre-incubation from cells differentiated using indomethacin and insulin when compared with those differentiated using dexamethasone, IBMX and insulin (glycerol release, 276.1 μg/ml, p= 0.065). Additionally, the increase in glycerol release that was stimulated by the adrenoceptor agonists in the indomethacin-differentiated adipocytes was similar when compared with the percentage changes observed for dexamethasone, IBMX and insulin treated cells. Pre-treatment with IL-6 for 16 h had no effect on lipolysis. Glycerol release from adipocytes differentiated using indomethacin and insulin, following 24 h pre-treatment in serum-free media, is shown in Figure 5.10. No inhibition is observed in the presence of IL-6, with the exception of CL316243-treated adipocytes, which have significantly reduced glycerol release when incubated with IL-6 [IL-6 treated vs. untreated; 305.2% (202.2–346.4) vs. 390.5% (375.5–467.1), compared with basal (no IL-6), p= 0.017].

Mechanisms of the IL-6 effect on lipolysis

To investigate the possibility that IL-6 was affecting lipolysis at the post-receptor level, possibly by altering the activity of HSL, adipocytes were incubated with the cell-permeable cAMP homologue dbcAMP, following 24 h of pre-incubation in the absence or presence of IL-6. There was no significant difference in glycerol release in the presence of IL-6 [IL-6 treated vs. untreated; 176.4% (166.2–177.7) vs. 170.1% (167.3–175.1) of unstimulated, p= 0.513].

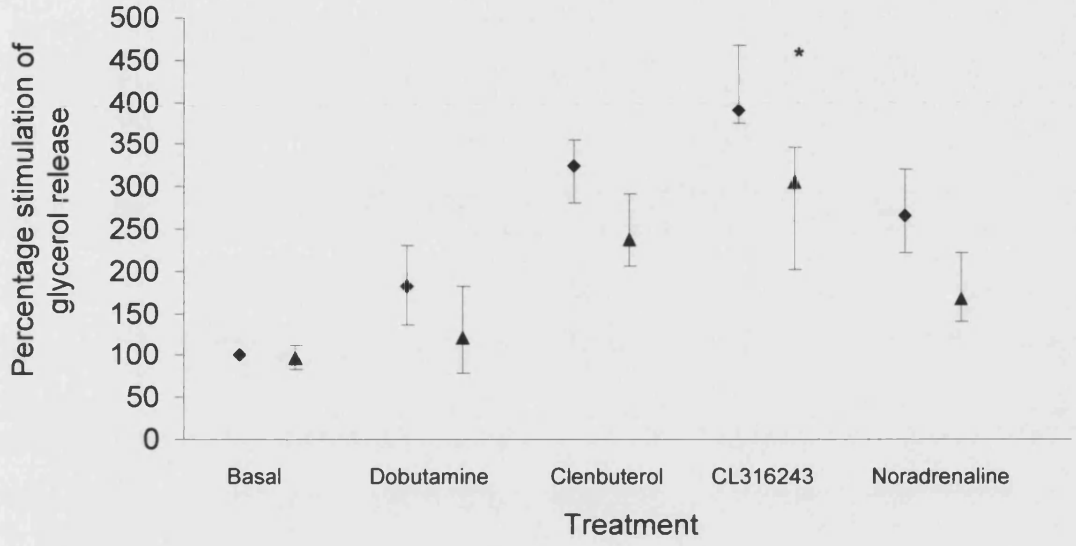

Figure 5.10 – Glycerol accumulation from 3T3-F442A adipocytes pre-treated for 24 h +/- IL-6, differentiated with indomethacin and insulin. The release of glycerol from 3T3-F442A adipocytes pre-treated in serum-free media for 24 h in the absence (♦) or presence (▲) of 10 ng/ml IL-6, prior to 6 h incubation with a β1- (dobutamine), β2- (clenbuterol), β3- (CL316243) or non-specific α/β-adrenoceptor agonist (noradrenaline), or no stimulant. Data presented as percentage stimulation (median and interquartile range) of glycerol accumulation compared with basal (no IL-6). Cells differentiated using indomethacin and insulin. *n*= 4 (no IL-6); *n*= 6 (+ IL-6).

**p*= 0.017.

Another possible mechanism is cytokine-induced alteration of β-adrenoceptor expression, which has been shown for TNFα [417]. Therefore, levels of adrenoceptor mRNA were examined. Probes for β2- and β3-adrenoceptors were used to measure the levels of the two receptors in adipocytes that were differentiated using dexamethasone, IBMX and insulin, and which had been pre-incubated for 24 h prior to the lipolysis experiments. There appeared to be no effect of IL-6 on receptor levels when measured by northern blot (Figure 5.11). The resolution of the blots made it difficult to make a quantitative assessment, however, so semi-quantitative real-time rt-PCR was used to measure the mRNA levels of the adrenoceptors. The expression of β2-adrenoceptors (Figure 5.12) in cells that were not treated with adrenoceptor agonists did not differ from the expression in cells that were treated with clenbuterol, either without [0.85 (0.52–1.2) fold (median and interquartile range) of basal levels, p= 0.423] or with [2.1 (0.6–2.5) fold, p= 0.337] IL-6 pre-treatment; expression levels following CL316243 treatment were similarly unaffected (without IL-6, 0.8 (0.7–0.9) fold, p= 0.078; with IL-6, 0.9 (0.7–1.5) fold, p= 0.522). There was also no difference in expression between IL-6 treated and untreated cells following either clenbuterol (p= 0.749) or CL316243 (p= 1) incubation. CL316243 treatment in the absence of IL-6 appeared to downregulate the expression of β3-adrenoceptors (Figure 5.13) compared with basal [0.36 (0.35–0.41) fold of basal, p= 0.01], although no significant differences were observed following the remaining treatments; clenbuterol [without IL-6, 1.2 (0.7–1.8), p= 0.631; with IL-6, 1.0 (0.6–1.3), p= 0.631], and CL316243 with IL-6 pre-treatment [0.9 (0.6–1.3), p= 0.749]. IL-6 treatment of the cells had no effect the expression of β3-adrenoceptors (clenbuterol, p= 0.631 and CL316243, p= 0.078).

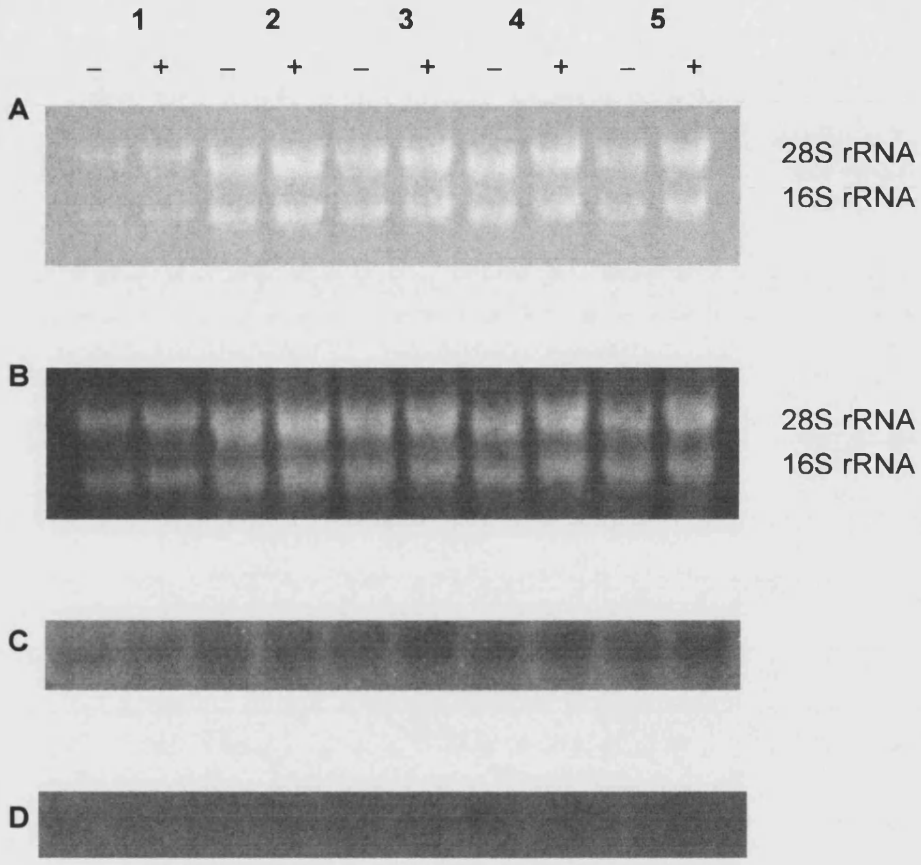

Figure 5.11 – Northern blots of β2- and β3- adrenoceptors.

Northern blots of β2- (**C**) and β3-adrenoceptors (**D**) following basal (**1**), and dobutamine (**2**), clenbuterol (**3**), dobutamine (**4**) and noradrenaline (**5**) stimulation, following 24 h pre-incubation in the absence (–) or presence (+) of 10 ng/ml IL-6. **A** shows the original RNA gel, and **B** the membrane to confirm transfer.

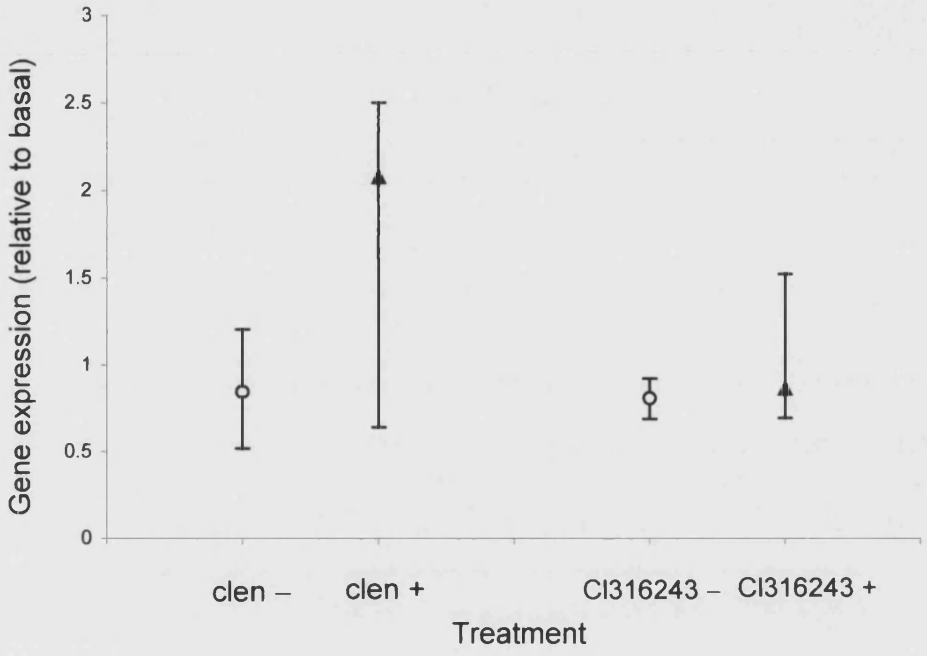

Figure 5.12 – Real time rt-PCR analysis of β2-adrenoceptor expression following IL-6 treatment.

The semi-quantative expression of β2-adrenoceptors following lipolysis stimulated with either clenbuterol (clen) or CL316243. Prior to incubation with the lipolysis agonists, adipocytes had been incubated in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels, and presented as fold change in expression compared with basal β2-adrenoceptor expression (not shown), median and interquartile range.

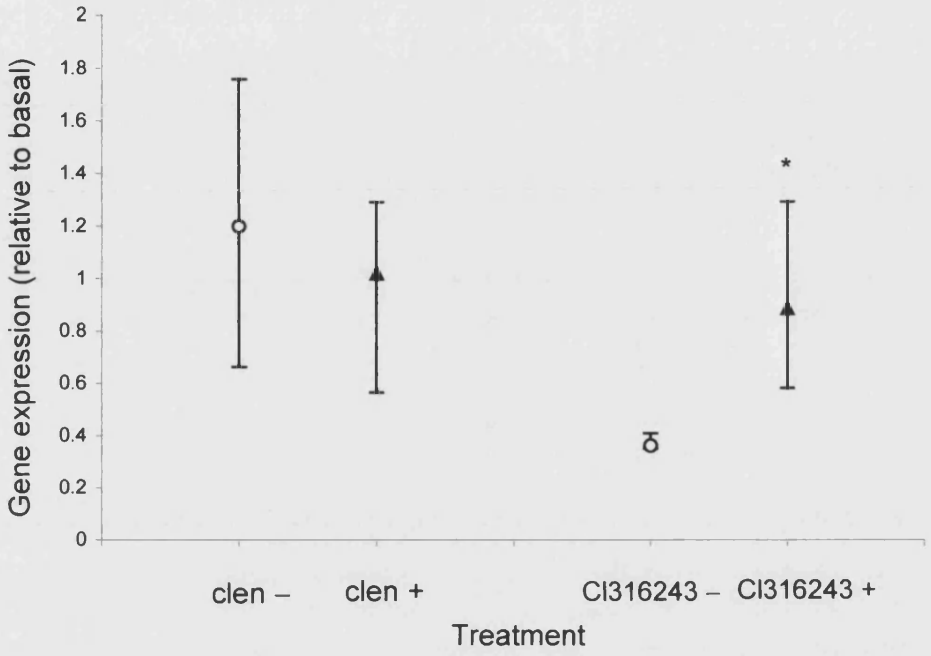

Figure 5.13 – Real time rt-PCR analysis of β3-adrenoceptor expression following IL-6 treatment.

The semi-quantative expression of β3-adrenoceptors following lipolysis stimulated with either clenbuterol (clen) or CL316243. Prior to incubation with the lipolysis agonists, adipocytes had been incubated in the absence (–, O) or presence (+, ▲) of 10 ng/ml IL-6. Expression is corrected to GAPDH mRNA levels, and presented as fold change in expression compared to basal β3-adrenoceptor expression (not shown), median and interquartile range.

*p= 0.01 compared with CL316243 no IL-6.

Discussion

As with the results from adipogenesis, the chronic incubation of differentiated adipocytes with IL-6 appears to have a lipid-enhancing effect. Both basal and insulin-stimulated glucose uptake was increased following treatment with IL-6, therefore increasing fat deposition within the adipocyte. Furthermore, the effect of β-adrenoceptor-related lipolytic signals is attenuated, preventing the loss of lipids from the adipocyte. These results, therefore, led to the immediate rejection of the initial TNFα-based hypothesis. This implicates IL-6 in the retention of adipose tissue, and might help to explain some of the observations from Chapter 4.

Owing to the strong correlations between adipokines and insulin resistance [161, 244, 254, 304, 418-422], there has been considerable interest in the effects of these cytokines on glucose uptake in the adipocyte. Early studies on adipocytes suggested, as the results here do, that the direct incubation of mature 3T3-L1 cells with IL-6 enhances glucose uptake [263], and also that this effect was not mediated via the traditional insulin-related pathway. Unfortunately, time constraints prevented a categorical answer to the question of whether IL-6 affects the intracellular trafficking of GLUT1. Total protein levels of either GLUT1 or GLUT4 remained unchanged in the presence of IL-6, but the increase in glucose uptake observed might have been due to an increase in GLUT1 at the plasma membrane.

No effect of IL-6 was observed following a more acute stimulation (less than 24 h) implying, as with adipogenesis, that only chronic incubation with IL-6 significantly affects adipocytes. This observation has been confirmed by a number of other studies [263-265]. These data also provide a mechanism for the differentiation-dependent effects of IL-6. Chronic incubation of mature adipocytes with IL-6 resulted in the increase of both basal and insulin-stimulated glucose uptake, and therefore lipid deposition. The occurrence of this during adipogenesis, although not

affecting the actual mechanism of differentiation, would increase the lipid contents within the differentiating cells. The features of IL-6 treatment that were observed during adipogenesis using dexamethasone, IBMX and insulin correspond to this: lipid vacuoles with a greater volume, and an increase in secreted leptin, which is related to lipid content of fat cells [34].

The difference in glucose uptake between 3T3-L1 adipocytes and 3T3-F442A adipocytes, and between the 48 h and 96 h insulin-withdrawal periods, might be due to a number of reasons. Concerning the period of insulin-withdrawal prior to the experiments, it must be noted that the concentrations of insulin used during the differentiation period is far higher than physiological levels could ever reach. Insulin has a potent downregulatory effect on the expression and protein levels of the glucose transporters and components of the insulin-signalling machinery [297, 423, 424]. Therefore, prior to measuring glucose uptake in these cells, this inhibitory effect of insulin must be negated. It is possible that, after 48 h, maximal basal function of the insulin-signalling system is not recovered, whereas 96 h is sufficient. The difference between the cell types might be because, although they are derived from the same source, they are fundamentally different for a number of physiological processes (also evidenced by the poor response of 3T3-L1 adipocytes for the measurement of lipolysis). For this reason alone, it is not surprising that the response to insulin might differ. It is also possible that the numbers of cells within the cultures could differ, although this has been corrected for as much as is possible in an adherent cell culture through the seeding of identical numbers of cells prior to growth and differentiation. Notably, it can be inferred that the 3T3-L1 cells are responding to insulin because the level of glucose uptake is similar to that observed in other studies [263].

The appearance of an almost synergistic response to glucose uptake in 3T3-L1 adipocytes treated with both IL-6 and insulin might be due to a number of factors. Whether the response is synergistic, and whether it is the result of transporter relocation to the plasma membrane

or increased GLUT activity cannot be concluded without further experimentation. However, the fact that the response was not observed in the less insulin-responsive 3T3-F442A cells leads to the possibility that insulin responsiveness has an effect on IL-6-induced glucose uptake in the presence of insulin (or, alternatively, on insulin-induced glucose uptake in the presence of IL-6).

These findings appear to challenge the reports of a number of studies concerning insulin resistance, which described the negative effects of IL-6 on glucose uptake. These range from associative studies, in which circulating IL-6 levels are positively correlated with numerous indices of insulin resistance [254, 255, 265, 304, 420, 421], to direct effects of IL-6 on cell lines and tissues [265, 421]. The first of these observations, concerning plasma IL-6 concentrations, in relation to studies on glucose clearance, circulating glucose levels and the effects of insulin, does not necessarily contradict the data presented here. There can be no dispute that IL-6 and insulin resistance are closely linked; adipose tissue releases IL-6 and this is closely related to body fat and obesity [103, 425-427], and obesity is a direct cause of insulin resistance [108, 316, 428-430]. If IL-6 increases glucose uptake, and thus lipid deposition, it is further contributing to excess fat deposition within the adipocyte and, therefore, indirectly causing insulin resistance. A further consequence of this is the release of more IL-6 from the excess adipose tissue, providing the circulating cytokine that enables these associations to be made. Whether IL-6 is inducing insulin resistance by other direct means, or through the IL-6-related involvement of other molecules, remains to be established.

IL-6 has also been reported to have a direct negative effect on adipocyte and other tissue glucose uptake [265, 421]. IL-6 appears to have no direct effect on glucose metabolism in muscle, at least with acute incubation [431], which is in agreement with the acute effects observed here. The effect on hepatocytes, in which the acute (< 1.5 h) incubation with IL-6 inhibits insulin action [421], might be ascribed to tissue specific differences. How to interpret the data obtained here in relation to the

report by Rotter *et al.* [265] is more difficult. Their data, based on similar methodologies applied here, suggest almost the opposite effects. Chronic (24 h) exposure of 3T3-L1 adipocytes to IL-6 resulted in the decrease of insulin-stimulated glucose uptake, as well as in the expression and protein levels of IRS-1 and GLUT4. Clearly, this is in direct conflict with the findings presented here, in which (in addition to the increase in basal and insulin-stimulated glucose uptake) IRS-1 and GLUT4 levels were unaffected. As with the data presented here, acute incubation with IL-6 had no effect. There are a number of differences between the experiments, although the relevance of these differences is questionable: The Rotter *et al.* [265] study used double the IL-6 concentration that was used in the studies presented here (20 ng/ml compared with 10 ng/ml), although both are within reasonable physiological concentrations for adipose tissue. In the data presented in this thesis, no effect of IL-6 was observed following 24 h pre-incubation, and subsequently no further investigation of this time-point was undertaken. Therefore, the effect of IL-6 on the GLUT and insulin signalling proteins was measured only after 48 h pre-incubation with IL-6. Finally the pre-treatment described in this study involved the complete removal of all serum and a defined minimal media (Cell-gro), whereas the Rotter *et al.* study used DMEM. Whether these differences are significant enough to explain the disparity in results cannot be known with the current data. The original intention of using different IL-6 concentrations might have helped to resolve this. Findings are further complicated by the fact that, independent of the apparent effects of IL-6 on glucose uptake in the tissues, there might be no net effect on total body glucose disposal caused by IL-6 [431].

Similar disparities in the effects of IL-6 on lipolysis in adipocytes have been published, although there is more variation in the methods used (compared with the relatively similar techniques that are used to measure glucose uptake). The data presented here indicate that, as with other effects of IL-6, acute incubation has no significant effect on adipocyte lipolysis, with one caveat. Following 12 h pre-incubation with IL-6, basal lipolysis was slightly, but significantly, increased. This is in

agreement with previous studies that have described an increase in basal lipolysis [267, 271]. Although this indicates that IL-6 might stimulate fat oxidation in adipocytes, the evidence is far from conclusive. As with the data presented here, which imply that an increase in glycerol release only results from an acute incubation with IL-6, neither of the two other studies reported an increase in lipolysis in all cells investigated. Mattacks *et al.* [271] noted that lipolysis was only stimulated by IL-6 in adipocytes from lymph nodes, which exhibit far lower levels of basal lipolysis than other adipocytes (and significantly greater responses to stimulators), and not from gonadal or perirenal depots. Furthermore, the action of IL-6 was modulated considerably by the co-incubation of IL-4, which resulted in an inhibition of glycerol release. This indicates that the source of adipocytes appears to be crucial in the effect of adipokines on lipolysis and must be considered in future studies. How the data from Path *et al.* [267] compare with that presented here is open to debate, because the 10 nmol/L IL-6 concentration they used was considerably greater than the 10 ng/ml (approximately 0.3 nmol/L) used here. Significantly, 24 h incubation of breast adipocytes with lower concentrations of IL-6 had no effect on lipolysis.

The fact that lipolysis is differentially affected following acute and chronic incubation is not necessarily paradoxical. IL-6 is released from muscle in response to exercise and is believed to maintain glucose homeostasis by inducing lipolysis [432, 433]. Notably, in both exercise and immune responses, the increase in IL-6 levels is large and transient. In these situations it might be beneficial to release substrate for energy production and IL-6 might be involved in the acute control of lipolysis in some tissues [434]. The situation in obesity is quite different to this; IL-6 levels are elevated but (at least in terms of initiating the acute phase response or activating immune responses) are subclinical. Furthermore, serum concentrations remain at these elevated levels chronically, as opposed to being cleared from the circulation within 48 h. This results in a very different response to IL-6, and could help to explain why studies have failed to pinpoint the precise role of IL-6 in adipocyte lipolysis. The

data presented here imply that, irrespective of its acute actions, chronic exposure to mildly elevated levels of IL-6 inhibits β-adrenoceptor-stimulated lipolysis. Whether this is a mechanism for maintaining stores of adipose tissue is unknown, but remains a possibility. Because basal lipolysis is not affected over longer time periods, these findings suggest that IL-6 is not directly affecting the lipolytic machinery within the adipocyte.

Unfortunately, attempts at identifying a definitive mechanism for the IL-6 effect on either glucose uptake or lipolysis were not completed. Possibilities were rejected, however: IL-6 does not affect glucose uptake by acting through IR or IRS-1 (although IRS-2 might be affected), or by increasing either GLUT1 or GLUT4 protein levels; the anti-lipolytic actions are not through the modulation of HSL function, because the IL-6 effect is cAMP insensitive, or by the downregulation of the β-adrenoceptors. Recent work in the laboratory has provided an interesting potential resolution, in combination with research published by other groups. The signalling molecule nitric oxide (NO) inhibits adrenoceptor-stimulated lipolysis in a similar manner to that of IL-6 in the data presented here [435, 436], without effects on basal lipolysis. Previous studies have suggested that NO can initiate the translocation of the insulin-dependent glucose transporter GLUT4 to the plasma membrane in the absence of insulin [437]. Preliminary data have suggested that IL-6 induces NO release in adipocytes (V. Mohamed-Ali, personal communication). If this is confirmed, and it is shown that IL-6 does initiate translocation of the insulin-sensitive glucose transporter GLUT4 to the plasma membrane, it would appear to be compelling evidence for a mechanism of chronic IL-6 stimulation of the adipocyte.

Chapter 6 – Conclusions and Future Directions

Adipokines and disease

Cytokines have been implicated in the pathogenesis of numerous diseases, in addition to their role in the immune response. Of note, obesity and its related disorders, including insulin resistance, are increasingly being correlated with adipose-tissue-derived factors. The direct mechanisms of how adipokines cause a disease state have yet to be established, although significant progress has been made in recent years concerning the effects of TNFα on insulin resistance [102]. Furthermore, correlations with insulin resistance have also been reported for IL-6, adiponectin, leptin and resistin [254, 438]. However, despite adipose tissue releasing considerable amounts of IL-6, especially in obesity [103], relatively little is known about its mechanism of action and its effects on adipose tissue.

Circulating IL-6 is correlated with obesity and insulin resistance, but the reported effects of IL-6 on muscle, adipose tissue and liver do not appear to implicate it in a mechanism for insulin resistance. Instead, the data presented here suggest that IL-6 does not have a direct effect on insulin resistance *per se*, but that it has profound effects on the biology of adipose tissue, particularly visceral adipose tissue, such that lipid retention is encouraged. From this pro-adipogenic state, insulin resistance and other pathologies are indirectly promoted.

The results from Chapter 3 showed that a proportion of IL-6 release from adipose tissue is constitutive, with considerable secretion in the absence of stimulation. This is not true for all tissue types that release IL-6; macrophages, skeletal muscle and liver all require stimulation to release appreciable amounts of IL-6. Furthermore, the constitutive release of IL-6 from adipose tissue increases in obesity, possibly reflecting changes in the composition of the tissue. Therefore, although the acute regulated release of IL-6 – which adipose tissue is also capable of – during infection or exercise is beneficial for anti-inflammatory and energy homeostatic effects, the constitutive release from adipose tissue is potentially harmful.

In Chapter 4, it was shown that IL-6 has a significant effect on lipid accumulation during adipogenesis. It must be noted that, although leptin release was increased and the adipocytes that were treated with IL-6 were larger than untreated cells, IL-6 was not directly affecting adipogenesis. Rather, IL-6 was promoting an increase in lipid deposition within adipocytes that were already committed to differentiation. Therefore, it appears that IL-6 is acting as a hypertrophy-promoting agent resulting in large adipocytes. This is important, because hypertrophic adipocytes are more likely to be involved in the pathogenesis of obesity-related disorders. Visceral adipose tissue correlates better with obesity-related disease states than does subcutaneous tissue [8, 439-441]. Previous studies have already implicated hypertrophy as the most significant factor in obesity (that is, the increased size of existing adipocytes is more significant in obesity and its diseases than is the differentiation of new adipocytes), although a degree of hyperplasia does occur [442-444]. This increased adipocyte size might also affect the production, release and function of other adipokines and cytokines – leptin release is significantly greater from large adipocytes, as has been shown in this study. Adiponectin, which appears to be protective against insulin resistance [445, 446], is reduced in obesity. Therefore, an increase in IL-6 and, subsequently, adipocyte size might affect insulin resistance indirectly, by inhibiting the production of adiponectin through a hypertrophy-related mechanism.

The studies described in Chapter 5 provide a possible mechanism for how IL-6 results in adipocyte hypertrophy. The chronic incubation of differentiated adipocytes with IL-6 results in an increase in both basal and insulin-stimulated glucose uptake and an inhibition of adrenoceptor-stimulated lipolysis. Both of these effects have serious implications for the lipid content of adipocytes because both will promote the production and retention of lipid within the cell. Therefore, the lipid deposition that was observed during adipogenesis in the presence of IL-6 might be attributable to these two mechanisms. Unfortunately, no molecular mechanism for this was obtained (although a number were ruled out). It is

possible that glucose uptake is enhanced by an increased translocation of the insulin-independent glucose transporter GLUT1 to the plasma membrane, although this remains to be determined. Again, the distinction between the acute and chronic effects of IL-6 must be defined. The high levels of circulating IL-6 that occur in acute disease responses and during exercise result in the release of lipids from stores within the liver and muscle [434, 447] (although even reports on the acute effects of IL-6 are conflicting: individual tissue depots might be affected by acute IL-6 infusion, but total body levels of glucose disposal might not be affected [431]). As presented here, the incubation of a (relatively) low level of IL-6 has no acute effect but reduces lipolysis and increases glucose uptake over a longer period of time.

Therefore, the release of IL-6 from adipose tissue can have serious consequences on the physiology of the adipocytes in the tissue, as a result of autocrine and paracrine responses. IL-6 is released constitutively in the absence of a signal, and this release is greater in the obese state. Because the adipocytes in this situation are surrounded by a chronically raised level of IL-6, they become further stimulated to store lipid: IL-6 increases basal (and insulin-stimulated) glucose uptake and inhibits lipolysis. One hypothetical possibility is that a positive-feedback mechanism of increased hypertrophy and increased secretion of IL-6 occurs, especially in visceral tissue depots. In this situation, IL-6 levels would be related to obesity, but the correlation that occurs with insulin resistance would be indirect, because IL-6 does not appear to have any direct negative effect on insulin action, especially in adipocytes. To test whether a positive-feedback mechanism occurs, experiments need to be performed that can differentiate between exogenously added IL-6 and that which is produced by the tissue being stimulated (for example, by labelling the exogenous IL-6 with a radioactive label that can be assayed separately and subtracting this measurement from the total IL-6 levels, or by adding a moiety to the exogenous protein so that it can be removed prior to assay, such as by filtering through antibodies directed against the moiety). Alternatively, an increase in adipocyte hypertrophy might have a

beneficial effect against insulin resistance; lipid storage in sites other than the adipocyte (such as the liver [448] or muscle [449]) are strongly correlated with complications such as insulin resistance and cardiovascular disease [450]. The prevention of this, through the preferential deposition of lipid into the adipocyte, might help to alleviate the severity of insulin resistance. However, this must be balanced against potential harmful effects, such as increased levels of adipokines that are related to insulin resistance, including TNFα. A third possibility is that IL-6 is acting as a macrophage factor in the adipose tissue. Infiltrated macrophages are likely to be activated, and the release of cytokines from macrophages [451] might further affect adipose tissue biology. Macrophage-derived products, particularly TNFα [452], could be involved in numerous adipocyte functions, including lipolysis [286] and apoptosis [93]. Furthermore, this might be one explanation for some of the anomalies observed in the effects of IL-6 on adipose tissue: (i) the incubation of IL-6 with adipose tissue, including resident macrophages, might affect the release of TNFα and the effects associated with it; (ii) some adipose tissue depots are less infiltrated by macrophages than others [23] and, accordingly, this might be responsible for the differential effect on different adipose tissues [267, 271]; and interventions on adipocytes in isolation, as reported here, would not be subject to these extra-adipocyte effects, and would only measure the direct effect of IL-6 on adipocytes themselves. Of course, these remain hypotheses and considerable work is required before the effect of IL-6 on adipocytes, and on adipose tissue, can be fully determined.

Overall, how does adipose-tissue (and adipocyte)-derived IL-6 fit into the scheme of adipokines and obesity-related disorders? As yet, there is too little information to make much more than educated guesswork; the data provided here give an idea of the effects of IL-6 in an autocrine/paracrine setting in adipose tissue, but also raise numerous questions and, in some cases, controversy. I would suggest that IL-6 has a pro-hypertrophic effect on adipocytes (in isolation), promoting the retention of lipid, and that it is a marker for adiposity in otherwise healthy

individuals. However, there are two significant caveats. First, IL-6 is unlikely to be as potent as, for example, TNFα with its effects on adipocytes. The IL-6-related responses are likely to be rather modest in comparison, without the dramatic immediate change in adipose tissue (i.e. de-differentiation [259] and apoptosis [93]) that follows TNFα incubation. However, because of the chronic timescales involved, it might have a longer-term effect on adipose tissue depot size and function, relating to an increase in adiposity over a period of months or even years. Second, the recent data concerning the composition of adipose tissue indicates that macrophages are likely to be important mediators of any adipose tissue effects involving inflammatory cytokines, such as IL-6. This might explain some of the differences observed in response to lipolysis (for example) between adipocytes alone and adipose tissue. Nonetheless, adipose tissue releases considerable IL-6, and the major differences observed in the responses of adipose tissue depots to IL-6 (both in this study and by Mattacks *et al.* [271], as well as the fact that IL-6-deficient mice are obese [274], indicate that IL-6 does have a role in adipose tissue biology. For these reasons, the effects of IL-6 on adipose tissue deserve further investigation.

Future directions

Adipose tissue releases numerous different adipokines, and these are regulated in many different ways. Studies on the individual products and their effects are ongoing, and knowledge of the interaction between them is relatively poor. Furthermore, although IL-6 is related to insulin resistance, it does not appear to directly affect insulin action. The data presented here suggest an indirect mechanism, possibly through one or more of the other adipokines. The metabolism of the adipocyte appears to be affected by the chronic influence of IL-6, and the molecular mechanisms causing this must be established to fully understand the autocrine and paracrine effects of IL-6 on adipocytes. The endocrine effects of adipocyte-derived IL-6, especially on the liver and skeletal muscle, need to be determined. Moreover, the interactions between adipocytes and the tissue immediately surrounding them could have a profound effect on the function of adipocytes, as shown by the effect of lymph nodes on adipose tissue physiology. It must also be noted that IL-6 might have a significant central effect on body mass. IL-6-knockout mice develop mature-onset obesity [274], which can be reversed by the central administration of IL-6 [275]. IL-6 levels in the central nervous system are inversely correlated with adiposity, the opposite of what is observed in the periphery [453]. Therefore, IL-6 released from adipose tissue must be considered as an autocrine, a paracrine and an endocrine and central signal in its effects on fat mass.

One important factor that must be determined is the actual composition of adipose tissue, especially the differences between the cellular constituents in the lean and the obese states. Studies have reported the infiltration of macrophages into adipose tissue in obesity [23]. Furthermore, an inflammatory state develops within the adipose tissue of the obese through changes in gene expression, resulting in macrophage-derived messages being expressed by adipocytes [24, 320]. Therefore, it is important to identify the cellular source of IL-6; because macrophages do not release significant quantities of IL-6 under basal

conditions, the release in lean animals might be solely adipocyte derived. During obesity, IL-6 might be released by adipocytes, by adipocytes with macrophage-like expression patterns or by infiltrating macrophages (which are likely to be activated and therefore releasing cytokines). The inflammatory-like state in obesity might also explain the increased sensitivity of adipose tissue to stimulators such as IL-1β and β-adrenoceptor agonists. The precise relationship between adipocytes and macrophages, and the possible interconversion between the two, with respect to dysfunction and cytokine release in obesity must be established.

Therefore, experiments similar to those performed here (in Chapter 3) should be undertaken on the individual separated fractions of adipose tissue, the adipocytes, immune cells and stromal vascular cells, along with the determination of the relative contribution of each. Of particular interest would be the investigation of adipocyte release of IL-6, in the absence of other adipose tissue cells. This would provide a clearer indication of the true importance of adipocyte-derived IL-6 within the adipose tissue milieu. At the biochemical level, it would be worthwhile defining further the mechanism of IL-6 secretion; considering that the process was shown here to be Golgi-dependent, its precise route from the cytoplasm to the plasma membrane would enhance the knowledge of adipocyte secretion pathways to add to the different ones already known for leptin and LPL. One possible mechanism of IL-6 release that deserves attention is the stimulation of the prostaglandin (PG) pathway. The pathway is involved in the release of IL-6 from macrophages [454]. Furthermore, IL-1β and stimulated β-adrenoceptors – both of which increased IL-6 release from adipocytes – can induce the production of PGE_2 [455-458] which, through interactions with its four cognate receptors (EP1–4), stimulates the induction of IL-6 [454].

In terms of the effects of IL-6 on adipocytes, the mechanisms of glucose uptake and lipolysis as a result of IL-6 must be established, and the effects of IL-6 during adipogenesis better understood. For the adipogenesis study, a repeat of the methodologies described here but

using gene microarray instead of PCR would provide a wealth of information on the changes in cellular expression that are related to the inclusion of IL-6 in the differentiation media. Of course, there are considerable cost implications for this approach, and real-time rt-PCR is an effective alternative. The use of end-point rt-PCR was shown to be less effective in this study; the difficulties in interpreting data when changes are minimal between two samples indicate its limitations. It remains a useful and cost-effective method for determining gross changes in expression (i.e. for studies that show a change from no expression in a system to measurable expression).

The glucose uptake studies, which confirmed previous reports of an increase in glucose uptake by IL-6, were, unfortunately, not completed in time. The subcellular distribution study of the GLUTs was a promising investigation into the mechanism of IL-6-induced glucose uptake. Similarly, investigations into the mechanism of IL-6 inhibition of adrenoceptor-agonist-induced lipolysis (such as NO signalling) is an important step in determining the true effect of IL-6 on lipolysis, for which there have been many conflicting reports. For completeness of the studies described, it would have been advantageous to investigate a number of different IL-6 concentrations, to determine how the adipocyte reacts (and possibly to explain some of the data reported here in comparison with data from other groups). The next stage, using these methodologies, would be to use primary fibroblasts as the precursor to the experiments, instead of the committed cell lines used here. Furthermore, glucose uptake and lipolysis measurements on explanted adipose tissue (from various depots), under the same conditions used here, would further delineate the true effect of IL-6 on adipose tissue, as well as the importance of the depot and the non-adipocyte cells.

Had the time and funds been available, one method that might have resulted in some persuasive data would be the use of transgenic animals. Two mouse models would make good starting blocks for the investigation of adipose-tissue-derived IL-6 on the phenotype: the lipodystrophic mouse and the IL-6-knockout mouse. By developing an

IL-6-overexpressing cell line (such as 3T3-L1s or 3T3-F442As) that can be grafted into the adipose tissue of the mouse, this will:

(i) For the lipodystrophic mouse, provide an *in vivo* in which the major adipose tissue sources (all implanted) are releasing excess IL-6. The effects on body composition can, therefore, be compared with mice transplanted with untransfected adipocytes.

(ii) Similarly, for IL-6-knockout mice, a similar comparison can be made *in vivo*, to measure the effects of adipose-tissue-derived IL-6 when it is the only source. This will be of particular interest to compare with the study of Wallenius *et al.* [275], to ascertain whether adipose-tissue-derived IL-6 can have central effects to reduce obesity, and if insulin resistance is improved or accentuated.

The results presented here have established that the mouse is a useful model for studying IL-6 in obesity. Using a knockout model, such as the leptin-deficient mouse used here, might be misleading in future studies, however, especially those investigating the whole-body effects of adipocyte-derived IL-6. Therefore, diet-induced obesity represents the best physiological model for studying obesity in the mouse to understand the mechanisms through which IL-6 has its effects. Translating these data to human obesity is, of course, desirable, but is unlikely to be realised in the immediate future. In the meantime, establishing the precise roles of IL-6 *in vitro* and in rodent models of obesity and adipose tissue will enable us to ascertain how harmful is the constitutive release of IL-6 from adipose tissue. The development of a specific inhibitor for adipocyte- and adipose-tissue-derived IL-6 would enable significant advances to be made in the study of IL-6 and obesity.

Appendix

Derivation of the real-time rt-PCR Method

The following describes the derivation of the $2^{-\Delta\Delta Ct}$ method for determining relative expression of genes, as developed by Livak and Schmittgen [295]. This is an abridged reproduction of the derivation described within that paper.

The exponential amplification of PCR is described as

$$X_n = X_o \times (1 + E_x)^n$$

Where: X_n is the number of target molecules at cycle n of the reaction

X_o is the initial number of target molecules

E_x is the efficiency of target amplification

n is the number of cycles

The threshold cycle (C_t) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. Thus,

$$X_T = X_o \times (1 + E_x)^{Ct,x} = K_x$$

Where: X_T is the threshold number of target molecules

Ct,x is the threshold cycle for target amplification

K_x is a constant

A similar equation for the endogenous reference (internal control gene) reaction is

$$R_T = R_o \times (1 + E_R)^{Ct,R} = K_R$$

Where: R_T is the threshold number of reference molecules

R_o is the initial number of reference molecules

E_R is the efficiency of reference amplification

Ct,R is the threshold cycle for reference amplification

K_R is a constant

Dividing X_T by R_T give the expression

$$\frac{X_T}{R_T} = \frac{X_0 \times (1+E_X)^{Ct,X}}{R_0 \times (1+E_R)^{Ct,R}} = \frac{K_X}{K_R} = K$$

The exact values of X_T and R_T depends on factors relating to the reporter dye used, but assuming efficiencies of the target and the references are the same,

$$E_X = E_R = E$$

$$\frac{X_0}{R_0} \times (1+E)^{Ct,X - Ct,R} = K$$

or

$$X_N \times (1 + E)^{\Delta Ct} = K$$

Where: X_N equals the amount of normalised target (X_o/R_o)

ΔCt equals the difference in threshold cycles for target and reference ($C_{t,X} - C_{t,R}$)

Which rearranges as

$$X_N = K \times (1 + E)^{-\Delta Ct}$$

Then, divide the X_N for any sample q by the X_N for the calibrator (cb), to give

$$\frac{X_{N,q}}{X_{N,cb}} = \frac{K \times (1+E)^{-\Delta Ct,q}}{K \times (1+E)^{-\Delta Ct,cb}} = (1+E)^{-\Delta\Delta Ct}$$

Such that $-\Delta\Delta Ct = -(\Delta Ct,q - \Delta Ct,cb)$.

For amplicons fewer than 150 base pairs, and in which primer and magnesium concentrations are optimised, the efficiency is close to one. Therefore, the amount of target, normalised to an endogenous reference and relative to a calibrator is given by

$$\text{Amount of target} = 2^{-\Delta\Delta Ct}$$

Example of the data obtained from real-time rt-PCR

Data for real-time rt-PCR are obtained automatically using the software for the PCR machine. The graph below is representative of the output from a real-time rt-PCR experiment (showing three samples, repeated in triplicate or quadruplicate), showing the change in relative number (y axis) during the amplification cycle (x axis). Each line represents the change in double-stranded DNA in one well, such that a complete 96-well plate would have data for 96 individual samples. One sample is a negative control (which does not rise far above the basal level) and the remaining two samples show a varying copy number.

When the PCR cycle has completed and a graph is obtained, an arbitrary value for a cut-off point must be chosen – the point at which all readings are taken. This cut-off must be in the linear phase of expansion of the samples, as early as possible into the exponential-growth phase. In the example below, a value of 1 on the y-axis might be used. Therefore, all measurements of Ct value, the cycle number at which the sample crosses the threshold, are taken when the sample reaches am absorption value of 1.

The Ct values obtained in this way are used in the calculations above to provide a relative amount of product that was present in the sample.

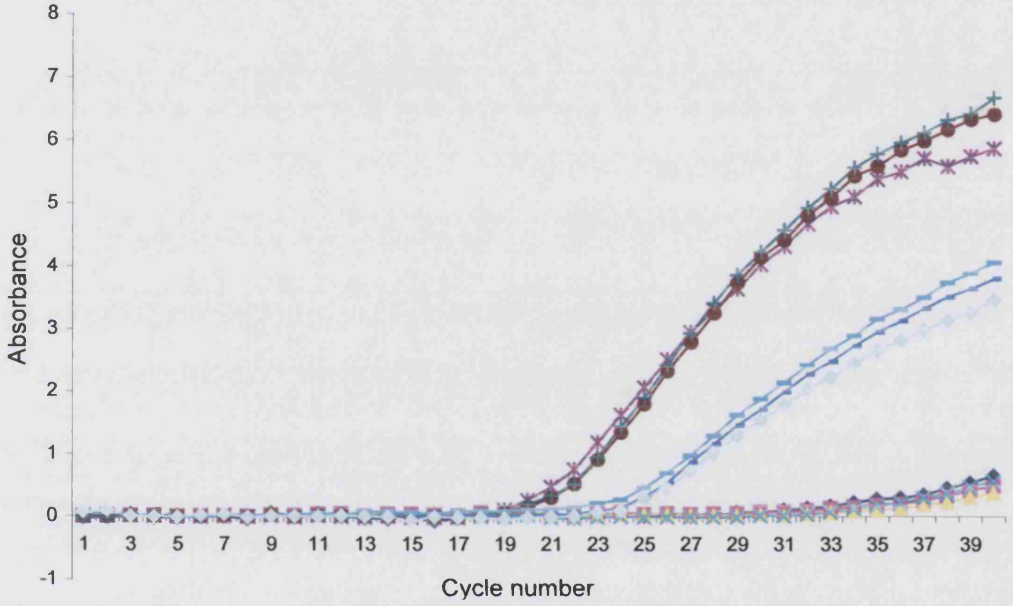

Figure I – Sample real-time rt-PCR graph.

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